
**EXPRESSION, PURIFICATION AND
CHARACTERISATION OF
PROTOPORPHYRINOGEN OXIDASES FROM
DIVERSE SPECIES**

by

Kwanele Bennett Siziba

**A thesis submitted in fulfilment of the requirements for the degree, Doctor of
Philosophy, in the Department of Medicine, Faculty of Health Sciences,
University of Cape Town.**

September, 2000

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

Acknowledgements

My gratitude goes to all the people for their material and spiritual support throughout my academic development. This thesis is a result of all the efforts put in by the various individuals.

Firstly, a special gratitude goes to my late parents, Willie Dingani and Nowinile Lettie Siziba for their belief and commitment to education, despite the fact that they never went to school. I am truly indebted to them for the sacrifices they made, and the wisdom they showed by leading me in this direction. I would also like to thank them for creating a family unit from which I was able to draw strength to carry on with whatever I was involved in. I am deeply grateful to my family, brothers and sisters, whose influence shaped my physical and spiritual development.

I wish to express deep and sincere gratitude to Professor Ralph Kirsch for accepting me in his laboratory at the MRC/UCT Liver Research Centre to do my PhD programme. His optimistic encouragement and constructive criticisms were invaluable in the development of this thesis. It has been a privilege and an honour to me to work with someone of his stature.

I am grateful for the support and guidance I received from Professor Pete Meissner and Dr Anne Corrigall, who supervised this work. My thanks goes to them for the training and support in developing the skills I acquired as a Medical Scientist, and also for their dedication and commitment in the preparation of this thesis.

My sincere appreciation also goes to Prof. Harry Dailey, Tammy Dailey and other members of their team, at the University of Georgia, Athens (USA), who engineered the human, *Bacillus subtilis* and *Myxococcus xanthus* recombinant protoporphyrinogen oxidases, and provided these for the characterisation conducted for this thesis. Collaboration with this team proved invaluable in terms of the useful and practical ideas which were exchanged.

I am deeply grateful to Nomsa Mda, a colleague and friend, who introduced and unselfishly encouraged me to do this Ph.D. programme. A special appreciation to Dr Matsiliza for introducing me also to this work and for being my mentor during this work at the MRC/UCT Liver Research Centre laboratories. To me they provided a family away from home.

I would like to acknowledge the contribution by Leslie Martin, Abduragman Damon, Mbulelo Maneli, Val Hancock and Lesley Frith in helping me perform the huge protoporphyrinogen oxidase kinetic assays. Without their assistance much of the findings of this study would never have been produced. A special

thanks to Mr Mick Wells who was always willing to help make and repair the equipment required for preparing the PPO substrate.

I would like to thank the staff at the UCT Electron Microscope Unit for helping and guiding me with the electron microscopy (E.M.) techniques, and use of their microscopes. Thanks to Mohamed Jaffer, Prof. Trevor Sewell, Mr William and Miranda Waldron for the useful discussions and practical assistance. I would like to thank Dr Richard Kirsch for the invaluable discussions and comments on the electron microscope studies, and my work in general. My thanks also goes to all the patients at the Liver Clinic (Groote Schuur Hospital) who gave informed consent and donated biopsy material for the E.M. studies. The experience of Dr Chick Campbell was invaluable in all the immunohistological studies.

On a number of occasions I benefitted also from the support and advice from several other members of the MRC/UCT Liver Research Centre to whom I am deeply grateful, and I wish to thank: Professors Enid Sherphard, Richard Hift, Pauline Hall and Phinda Songca; Drs Ed Sturrock, Henry Hairwadzi, Wendy Spearman, Susan Adams, Sharon Kelly and Vanessa Burch, and Lester Davids, Brent Jennings, Xolani William, Mark Kidd, Cornelius van der Hoft, Faiza Pearce, Lavinia Petersen, Mogamat Samuels, Fikile Daki, and Brandon Davids.

Lastly, a special gratitude to my children, family and friends at large who shared in every tribulation and triumph during the time I was doing the work that has provided the material for this thesis.

Abstract

Expression, Purification and Characterisation of Protoporphyrinogen Oxidases from Diverse Species

This work involved the characterisation of protoporphyrinogen oxidase (PPO), the penultimate enzyme in haem biosynthesis, from *Bacillus subtilis*, *Myxococcus xanthus*, and human. A defect in human PPO causes variegate porphyria, an autosomal dominant disorder characterised by skin photosensitivity and propensity towards acute neurovisceral crises. At the beginning of this project little information was available on the kinetic and biophysical properties of isolated PPOs due largely to difficulties associated with their purification from natural sources.

Recombinant PPOs of *B. subtilis*, *M. xanthus* and human, expressed in *E. coli* cells were purified to apparent homogeneity by a rapid metal chelate affinity chromatography procedure. These PPOs with subunit molecular weights ranging from 52000 to 54000 Da were shown to be FAD-containing flavoproteins. Optimal oxidation of the substrate, protoporphyrinogen-IX by the *B. subtilis* and *M. xanthus* PPOs occurred at pH 8.7 and 8.1, respectively. Whilst both the human and *M. xanthus* PPOs were highly specific for protoporphyrinogen-IX as substrate, the *B. subtilis* enzyme utilised both mesoporphyrinogen-IX and coproporphyrinogen-III as well. The fatty acids, oleic and palmitic, stimulated *M. xanthus* PPO activity, whereas the *B. subtilis* enzyme was inhibited. Inhibition studies enabled us to establish the inhibitor profiles of these PPOs. Acifluorfen and methylacifluorfen, which strongly inhibited both human and *M. xanthus* PPOs were ineffective against the *B. subtilis* enzyme. Human PPO was weakly inhibited by hemin and biliverdin, which strongly inhibited both *B. subtilis* and *M. xanthus* enzymes. At 100 μ M bilirubin did not inhibit human and *M. xanthus* PPOs, whilst approximately 95% inhibition of the *B. subtilis* enzyme was observed. This revealed interspecies differences in the behaviour of PPOs towards various compounds. In all cases effective inhibitors produced a competitive mode of inhibition with respect to protoporphyrinogen-IX.

Phylogenetic analysis using 28 different PPO protein sequences was performed, and interesting divergences amongst the prokaryotic forms of this protein were revealed. Immunocharacterisation studies with a polyclonal antibody raised against human PPO were used to demonstrate the differential expression of PPO in various human tissues/organs. Immunogold labelling and electron microscopy studies on human hepatocytes confirmed predominant location of PPO in mitochondria and also demonstrated cytosolic forms of PPO in these cells.

Kwanele Bennett Siziba

MRC/UCT Liver Research Centre

Department of Medicine, UCT Medical School

Observatory, 7925, Cape Town

ABBREVIATIONS AND SYMBOLS

g	gram	°C	degrees centigrade
mg	milligram	O.D.	optical density
µg	microgram	A	absorbance
ng	nanogram	kb	kilobases
L	litre	bp	base pairs
ml	millilitre	Da	Daltons
µl	microlitre	G	centrifugal force
M	molar (moles/litre)	rpm	revolutions per minute
mM	millimolar	vol	volume
µM	micromolar	α	alpha
nM	nanomolar	β	beta
nmol	nanomole	δ	delta
N	normality	∞	infinity
n	number of values used	BSA	bovine serum albumin
sec	second	V	volts
min	minute	kV	kilovolts
h	hour	mA	milliAmperes
cm	centimetre	W	Watts
mm	millimetre	R _f	relative migration value
mm ³	cubic millimetre	M _r	relative molecular weight
nm	nanometre	pI	isoelectric point
%	percent		

SDS-PAGE Sodium dodecyl sulphate
polyacrylamide gel electrophoresis

FAD flavin adenine dinucleotide

FMN flavin mononucleotide

RFU relative fluorescence unit

Å angstrom

His histidine

PCR polymerase chain reaction

PMSF phenylmethanesulfonylfluoride

IPTG isopropyl-1-thio- β -D-
galactopyranoside

DMSO dimethylsulfoxide

LB Luria-Bertani medium

PBS Phosphate buffered saline

EDTA Ethylenediethylaminetetra-
acetic acid

DPE Diphenyl ethers

TABLE OF CONTENTS

	PAGE No.
Chapter One: Literature Review	1
Haem Biosynthetic Pathway	1
Historical Background	1
Structure and Chemistry of Porphyrins and Porphyrinogens	3
Enzymology of Haem Biosynthesis	5
1. ALA Synthase [EC 2.3.1.37]	7
Regulation of Haem Biosynthesis in Non-erythroid and Erythroid tissue	9
2. ALA Dehydratase [EC 4.2.1.24] (Porphobilinogen Synthase)	10
3. PBG Deaminase [EC 4.3.1.8] (Hydroxymethylbilane Synthase)	13
4. Uroporphyrinogen Cosynthase [EC 4.2.1.75]	15
5. Uroporphyrinogen Decarboxylase [EC 4.1.1.37]	15
6. Coproporphyrinogen Oxidase [EC 1.3.3.3]	17
7. Protoporphyrinogen Oxidase (PPO) [EC 1.3.3.4]	19
8. Ferrochelatase (FC) [EC 4.99.1.1]	20
The Porphyrrias	23
Classification of Porphyrrias	24
Variegate Porphyria	27
History and Prevalence of Variegate Porphyria in South Africa	27
Presentation and Diagnosis of Variegate Porphyria	28
Clinical presentation	28
Biochemical presentation and diagnosis	29
Genetic presentation and diagnosis	30
General Comments	31
Chapter Two: Literature Review Continued	32
Protoporphyrinogen Oxidase (PPO) and the Conversion of Protoporphyrinogen-IX to Protoporphyrin-IX	32
Terminal Electron Acceptors in the Oxidation of Protoporphyrinogen-IX to Protoporphyrin-IX	33
pH Conditions for the Enzymatic Oxidation of Protoporphyrinogen-IX	34
Isolation and Purification of PPOs	35
PPO Protein Size Range	36
Subunit Composition of PPO Proteins	36
PPO Cofactor Composition	36
Substrate Specificity	39
Sub-cellular Localisation and Translocation of PPO	39
Effects of Various Detergents and Lipids on PPO Activity	42

Protoporphyrinogen Oxidase Inhibition Studies	43
I. Herbicidal PPO inhibitors	44
Resistance to herbicidal PPO inhibitors	47
II. Inhibition of PPO by haem and some of its metabolic products	48
III. Inhibition of PPO by compounds that inhibit flavoproteins	49
PPO Immunological Studies	50
The Cloning and Identification of PPO Genes	51
General Comments	54
 Chapter Three: Development of this Thesis	 55
Context in Which this Work was Performed	55
Previous Studies	55
The Project	55
 Chapter Four: Expression and Purification of the Recombinant Protoporphyrinogen Oxidases of <i>Bacillus subtilis</i>, <i>Myxococcus xanthus</i> and Human	 57
Introduction	57
Objectives	58
Methods and Materials	58
Cloning of the <i>B. subtilis</i> PPO gene	58
Transformation, Expression and Purification of the Recombinant <i>B. subtilis</i> PPO	58
Transformation of the PPO-containing plasmid into <i>E. coli</i>	58
Expression of the recombinant PPO	59
Purification	59
Purification of recombinant <i>B. subtilis</i> PPO using denaturing conditions	60
Partial purification of recombinant <i>B. subtilis</i> PPO using non-denaturing (native) conditions	60
Cloning, Transformation and Expression of the <i>M. xanthus</i> and Human PPOs	61
<i>M. xanthus</i> PPO	61
Human PPO	61
Purification of <i>M. xanthus</i> PPO	61
Purification of Human PPO	62
Results and Discussion	63

Concluding Remarks	69
Chapter Five: Characterisation of Protoporphyrinogen Oxidases of <i>Bacillus subtilis</i>, <i>Myxococcus xanthus</i> and Human	70
Introduction	70
Objectives	70
Methods and Materials	71
Determination of the relative M_r of the isolated <i>B. subtilis</i> PPO	71
Analysis of the subunit composition of the <i>B. subtilis</i> PPO	71
Determination of the pH optima of <i>B. subtilis</i> , <i>M. xanthus</i> and human PPOs	71
Determination of the rate constants, K_m and V_{max} , for the oxidation of protoporphyrinogen-IX by the different PPOs	72
Determination of the isoelectric point (pI) of the purified <i>B. subtilis</i> PPO	72
Amino acid composition of <i>B. subtilis</i> PPO	72
Determination of substrate specificity of the recombinant <i>B. subtilis</i> PPO	73
Analysis of the flavin cofactors associated with <i>B. subtilis</i> and <i>M. xanthus</i> PPOs	73
Effects of various lipids on activity of <i>B. subtilis</i> and <i>M. xanthus</i> PPOs	74
Results and Discussion	74
Concluding Remarks	90
Chapter Six: Inhibition Studies on <i>Bacillus subtilis</i>, <i>Myxococcus xanthus</i> and Human Protoporphyrinogen Oxidases	91
Introduction	91
Objectives	91
Methods and Materials	91
Inhibitor assay preparation	91
PPO assay in the presence of inhibitors	92
Derivation of the constants	92
Definitions	93
Results	93
Discussion	99
Concluding Remarks	103

Chapter Seven: Phylogenetic Analysis of Protoporphyrinogen Oxidases Using Protein Sequences	104
Introduction	104
Objectives	105
Methods and Materials	105
Identification and Retrieval of PPO Sequences from Genbank TM and TIGR Databases	105
Multiple Sequence Alignment and Phylogenetic Analysis	106
Results and Discussion	107
Concluding Remarks	115
 Chapter Eight: Immunocharacterisation of Human Protoporphyrinogen Oxidase	 116
Introduction	116
Objectives	117
Methods and Materials	117
Antibody production	117
Light Microscopy – Determination of the expression of PPO in various human tissues	117
<i>Tissue specimens</i>	117
<i>Immunohistochemistry</i>	117
Electron Microscopy – Determination of the sub-cellular distribution of PPO in human hepatocytes	118
<i>Liver tissue specimens</i>	118
<i>Specimen preservation and cryoultramicrotomy</i>	118
<i>Immunogold labelling</i>	118
Results	120
Discussion	123
Concluding Remarks	126
 Chapter Nine: Conclusions and Future Directions	 127
 Appendices: Experimental Methods and Materials	 129
 References	 162

Chapter One

Literature Review

Haem is an iron-containing complex of protoporphyrin which associates with several proteins and is central to virtually all biological oxidations. In all living organisms haem is synthesised via a specific pathway involving a series of chemical reactions and modifications of various porphyrin intermediates (see below).

While this work is primarily concerned with detailed study of one specific protein, the penultimate enzyme of haem biosynthesis, protoporphyrinogen oxidase (PPO), this chapter serves to describe the context within which PPO can be viewed. It will cover general aspects of haem biosynthesis, the enzymes of haem biosynthesis and the porphyrias (especially variegate porphyria). PPO itself is reviewed in necessary detail in Chapter 2.

Haem Biosynthetic Pathway

Historical Background

An historical perspective of the porphyrins and haem biosynthesis allows some useful insight into the processes which have resulted in the development of knowledge in this area. The existence of porphyrins was first alluded to by Scherer in 1841 when he showed that iron was not responsible for the red colour of blood. He demonstrated the existence of the red colour following the washing away of precipitated iron from the solution. Similar demonstrations were done in experiments conducted by other researchers (Lecanu, 1837; Berzelius, 1840; Mulder, 1844). The substance responsible for the red colour was subsequently named "cruentine" by Thudichum (1867) who also defined its visible spectrum. The iron-free "purple-red fluid" described by Mulder (1844) in his experiments was later found by Hoppe-Seyler (1871) to consist mainly of a substance he called "hematoporphyrin". Hoppe-Seyler (1879) rediscovered the red fluorescence in his study of the porphyrin in chlorophyll, and he named it phylloporphyrin. The word porphyrin is derived from the Greek word *porphuros*, viz. purple.

Schultz (1874) described a 33 year old patient suffering from skin photosensitivity from the age of three, who passed wine red urine. On investigation the urine was found to contain a pigment with a spectrum similar to that of hematoporphyrin (Baumstark, 1874). Importantly, in the interpretation of these findings Baumstark (1874) suggested that these urinary porphyrin pigments were originating from an "error of synthesis", a fact which was confirmed fifty years later. A dark pigment isolated from a rheumatic patient treated with salicylates was described by MacMunn (1880) and he named it "urohematin", and he later renamed it "urohematoporphyrin" because of its striking resemblance to haematoporphyrin (MacMunn, 1885). Subsequently, this pigment was found in association with several other diseases as well (Le Nobel, 1887).

Nencki and Sieber (1888) showed that haematoporphyrin was a dicarboxylic porphyrin which could undergo esterification at carboxyl groups. Coproporphyrin was first prepared from urine by Sallet (1896) and was named “urospectrine”. This compound was later correctly named by Fischer and Zerweck (1924). Protoporphyrin was also identified around this time (Laidlaw, 1904). Subsequently, the correct tetrapyrrole structure of haem was proposed by Kuster (1912). However, at that time other scientists had reservations about the stability of such a large ring structure. The remarkable work by Fischer (1915) and other workers resulted in the differentiation of the natural haem from haematoporphyrin which was correctly named, protoporphyrin. The terminology used for the tetrapyrrole biosynthetic pathway intermediates, the porphyrins, was applied to the description of diseases of the haem pathway in 1937 – they became known as “the porphyrias” (Waldenstrom, 1937).

The isolation of crystalline porphobilinogen (PBG) from the urine of acute intermittent porphyria patients was another important milestone in the understanding of this pathway (Westall, 1952). Cookson and Rimington (1954) elucidated the monopyrrole structure of PBG. This compound (PBG) was shown to be enzymatically converted into uroporphyrinogen when incubated with chicken red cell haemolysate (Falk et al, 1953). Attempts to study the tetrapyrrole biosynthetic pathway using uroporphyrin-III which had been prepared from turacin (a copper complex of uroporphyrin-III found in the feathers of a bird species, the Cape Loerie) by the removal of the bound copper ions were not successful (Rimington, 1939). The reasons for this only became clear when it was realised that the actual intermediate substrates of the tetrapyrrole pathway reactions were porphyrinogens, the reduced forms of porphyrins (Bogorad, 1955).

Studies carried out in suitable animal models provided important information on the systematic biochemical description of the haem (tetrapyrrole) biosynthetic pathway. Various investigators studied the incorporation of radiolabelled precursors at different steps of the pathway during the formation of the haem molecule. Initial studies described how [^{15}N]glycine and [^{14}C]glycine were incorporated into haem in humans and other animals (Shemin and Rittenberg, 1946; Shemin et al, 1955; Gray, 1952; Grinstein et al, 1950; Muir and Neuberger, 1950). Labelling studies with [^{14}C]acetate (Shemin and Wittenberg, 1951) and *in vitro* experiments in avian erythrocyte preparations established that succinyl-CoA was the 4-carbon compound from the tricarboxylic acid cycle which provided some of the carbon atoms in the haem macrocycle (Gibson et al, 1958). Subsequently, it was demonstrated that 5-aminolaevulinic acid (ALA) was the committed precursor leading to the synthesis of all the porphyrin intermediates (Shemin and Russell, 1953; Neuberger and Scott, 1953). During this same period it was shown that PBG is the monopyrrole precursor for all the tetrapyrrole synthesis in this pathway (Falk et al, 1953). Thus, the porphyrin biosynthetic pathway was established by the mid-50's and was shown to be virtually the same as that described earlier by Lemberg and Legge (1949).

Structure and Chemistry of Porphyrins and Porphyrinogens

The structural and physical chemistry of porphyrins is covered in several earlier reviews (Burnham, 1968; Marks, 1969; Adler, 1973; Smith, 1975; Dolphin, 1979; Meissner, 1990; Moore, 1990; McDonagh and Bissell, 1998). Essentially, porphyrins are tetrapyrroles which consist of four weakly aromatic pyrrole rings that are linked by four methene bridges (-CH=). The four pyrrole rings have been designated A, B, C, and D, starting at the top and proceeding clockwise (figure 1.1) (Falk, 1964).

The four methene bridges are designated α , β , γ , and δ in the same order as the rings (Fischer and Orth, 1934). The porphyrinogen macrocycle is a rigid planar structure with eight positions where substituents (side chains) can be attached. Irradiation of the porphyrins with ultraviolet light at a wavelength of approximately 400nm results in red fluorescence, indicating the high degree of conjugation of these macrocycles. This property of the porphyrins was first described for haemoglobin by Soret (1883), and this absorption band is referred to as the Soret band. The side chain substituents attached to the rings are important in determining the physical characteristics of the porphyrins. As biological intermediates, the porphyrins exist as the unconjugated, less stable reduced forms, the porphyrinogens. Oxidation of a porphyrinogen will produce the corresponding porphyrin. Porphyrinogens are relatively non-aromatic compounds, with less rigid structural properties (Smith, 1975), and they do not fluoresce under ultraviolet light. Indeed, the oxidation of protoporphyrinogen to protoporphyrin is carried out *in vivo* by the enzyme PPO, the subject of interest to this thesis.

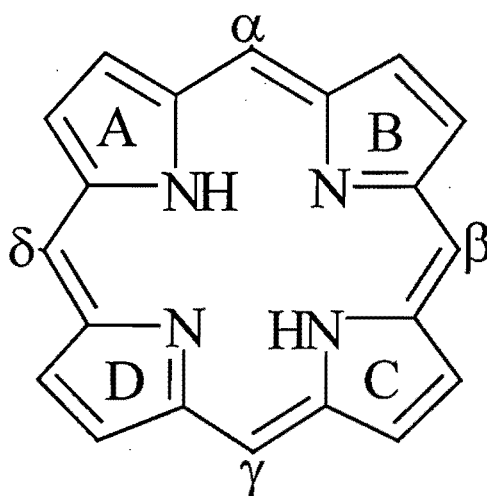


Figure 1.1 The tetrapyrrole structure of porphyrins showing the four pyrrole rings joined by methene bridges.

An important property of the porphyrin macrocycle is the availability of ligand binding sites. This gives these compounds the ability to bind metals; particularly iron to form haem, magnesium to form chlorophylls and cobalt to form vitamin B₁₂ via the corrins (Scott et al, 1972; Battersby and McDonald, 1975; Jones, 1976; Bissell and Schmid, 1987). In all living organisms the porphyrin biosynthetic pathway leads to the production of haem, and chlorophyll formation in photosynthetic organisms. As

mentioned in the introduction, haem (figure 1.2) associates with several proteins and is central to virtually all biological oxidations. Haem, by virtue of its ferrous iron being coordinately bound within the tetrapyrrole ring, plays specific roles in oxygen binding, electron transport, reduction of oxygen and transfer of oxygen for hydroxylation reactions.

These roles are in turn determined by the structure of the protein moiety of each specific haemoprotein, their substrates, and the intracellular milieu within which it functions (Bottomley and Muller-Eberhard, 1988). Chlorophylls, on the other hand, (figure 1.3) are magnesium-porphyrin complexes which are central in the utilisation of solar energy through photosynthesis.

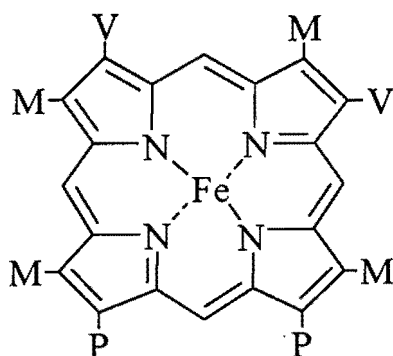


Figure 1.2 The tetrapyrrole structure of the haem molecule showing the ferrous iron (Fe^{2+}) in the centre. M = methyl ($-\text{CH}_3$), V = vinyl ($-\text{CH}=\text{CH}_2$), and P = propionate ($-\text{CH}_2\text{CH}_2\text{COOH}$) groups.

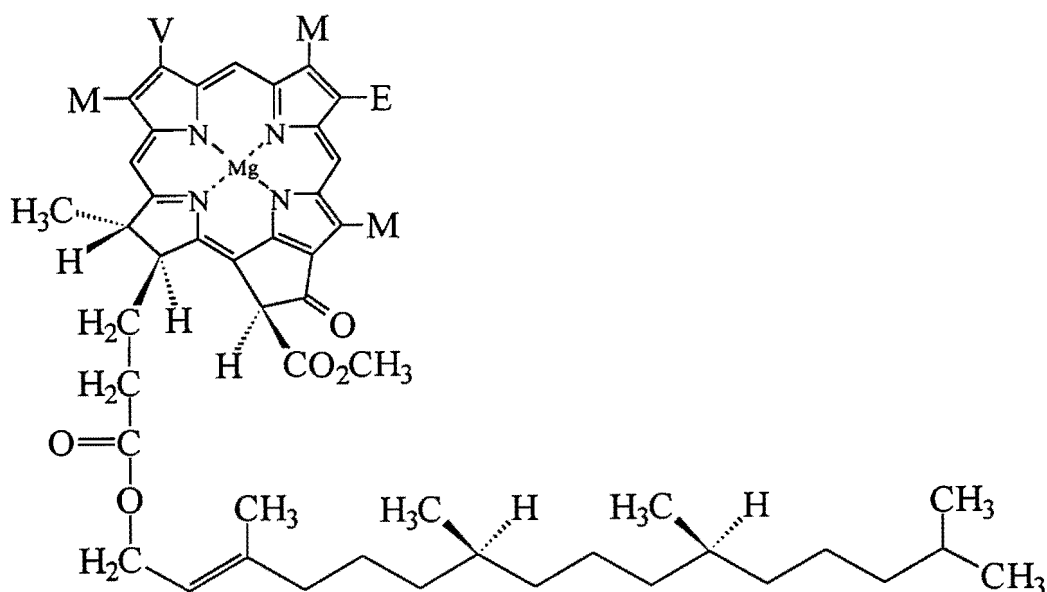


Figure 1.3 The structure of chlorophyll a, with the magnesium ion in the centre of the tetrapyrrole ring. M = methyl ($-\text{CH}_3$), V = vinyl ($-\text{CH}=\text{CH}_2$), E = ethyl ($-\text{CH}_2\text{CH}_3$) and P = propionate ($-\text{CH}_2\text{CH}_2\text{COOH}$) groups.

Enzymology of Haem Biosynthesis

Haem biosynthesis can be viewed in the most general terms as consisting of a series of "pyrrolic" chemical reactions and modifications catalysed and linked by the so-called haem biosynthetic enzymes, forming an irreversible, unbranched metabolic pathway. The enzymes of the pathway are located sequentially in the mitochondria, cytoplasm, and finally mitochondria (Dailey, 1990). The synthesis of chlorophyll on the other hand occurs exclusively within the plastids (Beale and Wenstein, 1990) as demonstrated by the experiments which showed the synthesis of chlorophyll in isolated chloroplasts from [^{14}C]ALA or [^{14}C]glutamate (Fuesler et al, 1984; Gomez-Silva et al, 1985; Huang and Castelfranco, 1988).

In a wide variety of organisms the sequence of reactions in this pathway is highly conserved after the initial reactions leading to ALA synthesis (figure 1.4) (Jordan, 1990). Both photosynthetic and non-photosynthetic organisms share a common pathway and generally have identical enzymes from ALA to the formation of protoporphyrin-IX. In animals ALA, the first committed precursor, is synthesized in a single reaction from glycine and succinyl-CoA by the mitochondrial matrix enzyme ALA synthase [E.C. 2.3.1.37]. In plants and some algae ALA is synthesized from the intact carbon skeleton of glutamate in three sequential enzymic steps via what is referred to as the C₃ pathway (Beale and Castelfranco, 1974a; Gough et al, 1976; Oh-hama et al, 1982; Porra et al, 1983; Wang et al, 1981; Kannangara et al, 1988).

The enzymic description of the haem biosynthetic pathway drew much interest when in the mid-50's Goldberg et al (1956) described ferrochelatase, the enzyme which catalyses the incorporation of ferrous iron into the protoporphyrin macrocycle to form protohaem. PPO, which catalyses the penultimate step in the haem synthesis pathway, was the last enzyme of the pathway to be described (Jackson et al, 1974; Poulson and Polglase, 1975) and it is the least characterised of these enzymes, motivating, at least in part, our interest in this protein.

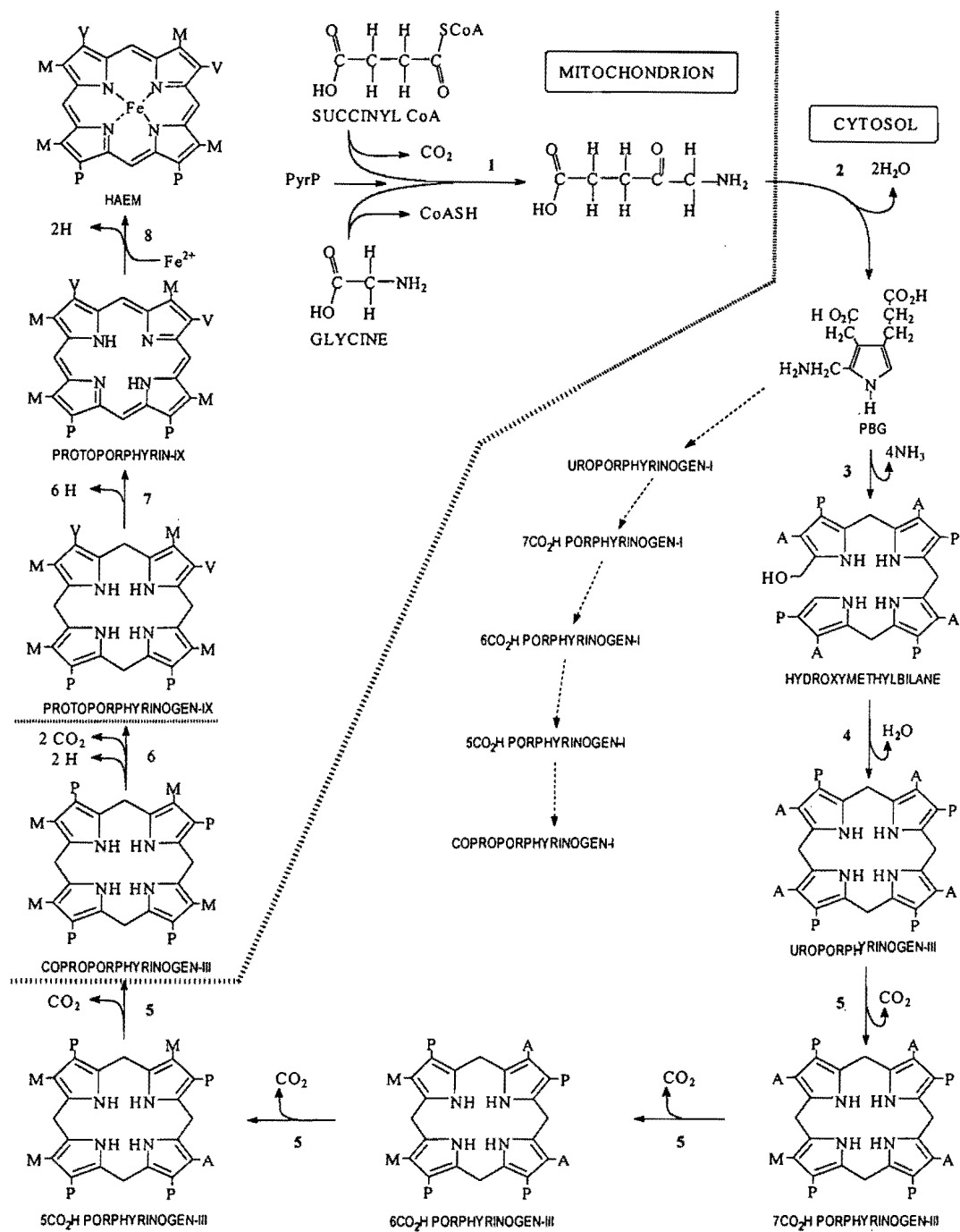


Figure 1.4 Haem biosynthesis involves eight enzyme catalysed "pyrrolic" reactions which are located sequentially, in the mitochondria, cytosol and finally mitochondria. M = methyl (-CH₃), V = vinyl (-CH=CH₂), P = propionate (-CH₂CH₂COOH), and A = acetate (-OCH₃). 1: ALA Synthase; 2: ALA Dehydratase; 3: PBG Deaminase; 4: Uroporphyrinogen Cosynthase; 5: Uroporphyrinogen Decarboxylase; 6: Coproporphyrinogen Oxidase; 7: Protoporphyrinogen Oxidase; 8: Ferrochelatase.

1. ALA Synthase [EC 2.3.1.37]

The first reaction of the haem biosynthesis pathway takes place in the mitochondrion. This involves the condensation of succinyl-CoA and glycine to form 5-aminolaevulinic acid (ALA) (1st reaction, figure 1.4), and is considered the rate-controlling step of the pathway (Gibson et al, 1958; Kikuchi et al, 1958a, b; Granick, 1963; Kappas et al, 1989).

This reaction is catalysed by the enzyme ALA synthase which is loosely bound to the inner mitochondrial membrane (McKay et al, 1969). In some early studies small amounts of this enzyme were found in the cytosol and were presumed to be newly synthesized protein destined for mitochondrial import (Hayashi et al, 1969; Patton and Beattie, 1973). Studies following this showed that the ALA synthase protein is encoded by nuclear DNA and synthesized in cytoplasmic ribosomes as a larger precursor protein before its final insertion into the mitochondrial membrane. There it is located as a modified smaller mature protein (Hayashi et al, 1970, 1976; 1983; Whiting and Elliott, 1972; Yamauchi et al, 1980; Borthwick et al, 1985; Urban-Grimal et al, 1986). Studies carried out on mitoplasts using specific enzyme markers, and *in vitro* investigations of the rat enzyme showed existence of this protein in the mitochondrial matrix (Scotto et al, 1983).

This protein has been purified from several sources including *Rhodobacter sphaeroides* (Warnick and Burnham, 1971); rat liver (Ohashi and Kikuchi, 1979); chicken liver (Borthwick et al, 1983); *Euglena gracilis* (Dzelzkalns et al, 1982) and yeast (Volland and Felix, 1984). The isolated proteins exist as homodimers with subunit molecular weights ranging from 40 000 to 70 000 Da, although it is now accepted that the mature mitochondrial forms of human erythroid and housekeeping ALA synthases have molecular weights of 59 500 Da and 64 600 Da, respectively (Cox et al, 1991).

This enzyme requires pyridoxal phosphate as an essential cofactor for activity (Granick and Sassa, 1971, Jordan and Shemin, 1972). The proposed mechanism of action is that glycine, bound through pyridoxal phosphate as a stable Schiff-base carbanion on the enzyme surface, can react with the electrophilic carbonyl group of succinyl CoA to produce an α -amino- β -keto adipic acid with the release of coenzyme A, which is then decarboxylated to ALA (Moore et al, 1987). Studies on the mouse enzyme have demonstrated a Schiff base linkage between the pyridoxal phosphate cofactor and a lysine residue (Lys-313) in the protein (Ferreira et al, 1993). Site-directed studies revealed that this lysine residue was essential for catalytic activity of the enzyme, and mutant proteins were shown to be able to bind the pyridoxal phosphate cofactor, although this binding was found to be non-covalent (Ferreira et al, 1995).

Application of molecular biology techniques has resulted in the cloning and sequencing of ALA synthase genes from several sources which include chick liver (Borthwick et al, 1985; Maguire et al, 1986; Yamamoto et al, 1985); yeast (*Saccharomyces cerevisiae*) (Urban-Grimal et al, 1986); human liver (Bawden et al, 1987); mouse (Schoenhaut and Curtis, 1986); *B. japonicum* (McClung et al, 1987); *E. coli* K12 strain (Li et al, 1989; Verkamp and Chelm, 1989); *Salmonella typhimurium*

(Elliott, 1989); *Aspergillus nidulans* (Bradshaw et al, 1993); rat liver (Yomogida et al, 1993); *Rhodobacter sphaeroides* (Neidle and Kaplan, 1993) and *Chlorobium vibrioforme* (Majumdar et al, 1991). Interestingly, comparison of the primary sequences of the human with the rat and chicken sequences revealed 83% and 87% similarities, respectively, but only 43% similarity with the mouse sequence (Jordan, 1990). The cDNA encoding the human enzyme had an N-terminal leader sequence of 56 amino acids (Bawden et al, 1987).

It is now recognised that there are two separate genes encoding ALA synthase. There is an ubiquitously expressed "housekeeping" (nonerythroid) isoenzyme (ALA-S1) and an erythroid-specific isoenzyme (ALA-S2) (Riddle et al 1989; Bishop, 1990; Cox et al, 1992; Conboy et al, 1992). Although the ALA-S1 and ALA-S2 isoforms share over 50% amino acid identity, they are localized to chromosomes 3p21 and Xp11.21, respectively (Bishop et al, 1990).

It was proposed that the reduced activity of ALAS2 in X-linked sideroblastic anaemia could be due to mutations in the erythroid-specific ALA synthase (ALAS2) gene (Bishop et al, 1990). Indeed, this was confirmed in a patient with X-linked sideroblastic anaemia by the identification of a T>A transition (codon 471/exon 9, a highly conserved region) causing an Ile>Asn substitution which interrupted contiguous hydrophobic residues (Cotter et al, 1992). The amino acid substitution was apparently in the putative pyridoxal 5'-phosphate binding site and structural prediction suggested that it caused a transformation of beta-sheet structure into random-coil structure. The mutant construct expressed in prokaryotic cells was shown to have low levels of enzymatic activity which required higher concentrations of pyridoxal phosphate to reach maximal activity compared to the normal enzyme. A number of mutations have been identified in the ALAS2 gene of different patients with pyridoxine-responsive, X-linked sideroblastic anaemia (Cotter et al, 1994, 1995; 1999, Edgar et al, 1997, 1998; Edgar and Wickramasinghe, 1998; Furuyama et al, 1997, 1998; Harigae et al, 1999a, and 1999b). Addition of pyridoxal 5'-phosphate *in vitro* to the purified mutant recombinant enzymes caused their activation and/or stabilisation, a fact consistent with the observed pyridoxine-responsive sideroblastic anaemia *in vivo* (Cotter et al, 1995).

The synthesis of ALA in plants, algae and some photosynthetic bacteria occurs by a different pathway involving the carbon skeleton of glutamate (Beale and Castelfranco, 1974a; Beale et al, 1975). This reaction occurs via the formation of an intermediate, glutamate 1-semialdehyde which is transformed into ALA by an aminotransferase (Houen et al, 1984) (figure 1.5). It has been proposed that in plant systems glutamate 1-semialdehyde undergoes transformation into another intermediate; 4,5-dioxovalerate (DOVA) before the final formation of ALA (Beale et al, 1975; Breu et al, 1988; Breu and Dornemann, 1988). This intermediate (which has also been found in bovine liver extracts (Varticovski et al, 1980)) is converted by the enzyme 4,5-DOVA transaminase into ALA (Morton et al, 1981). The three enzymes involved in transforming glutamate into ALA have been characterised, and they are glutamate-tRNA ligase (Bruyant and Kannangara, 1987), NADPH-dependent enzyme (Houen et al, 1984), and glutamate 1-semialdehyde aminotransferase (Hoover et al, 1988). It is widely accepted that the primary mechanism of regulation of haem synthesis is modulated by ALA synthase. This is therefore considered in some detail below.

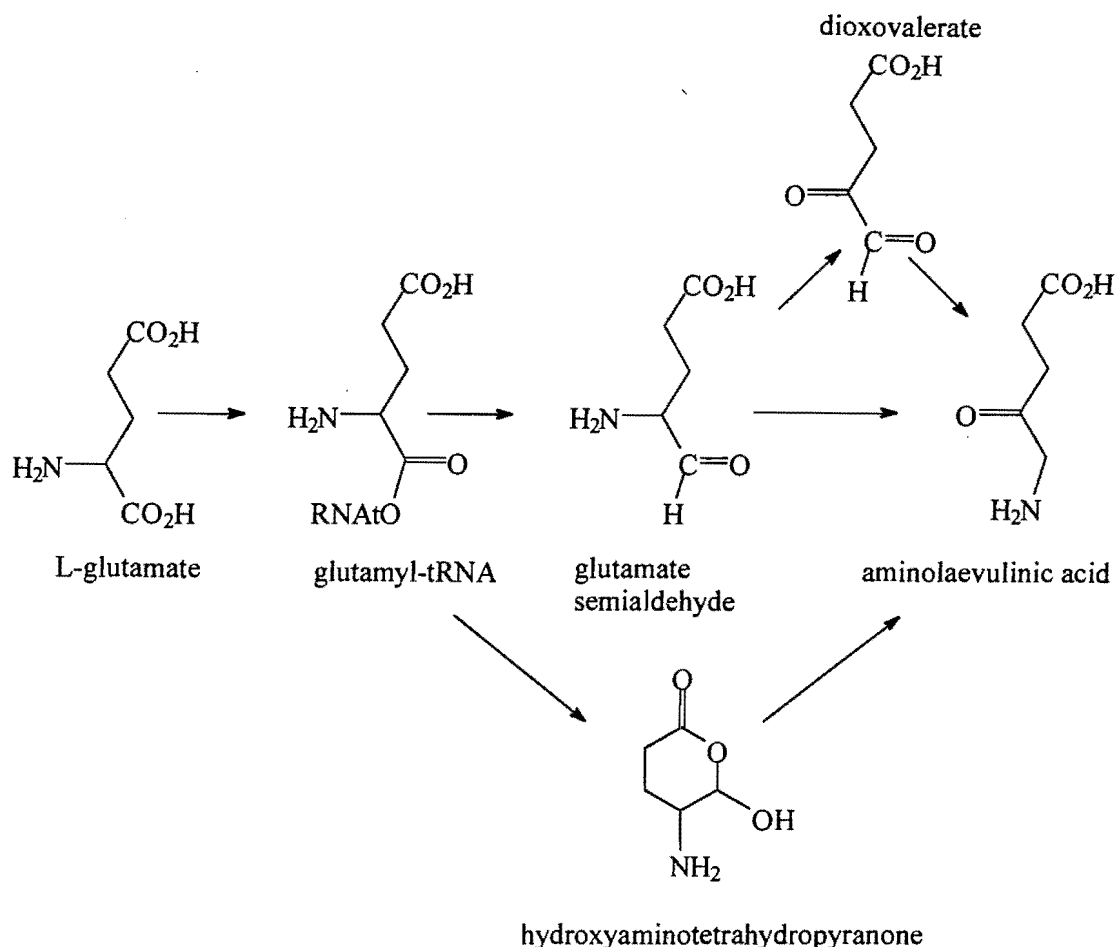


Figure 1.5 Formation of aminolaevulinic acid in plants and green algae showing the various reactions and intermediate products.

Regulation of Haem Biosynthesis in Non-erythroid and Erythroid tissue

Haem synthesis is normally an extremely efficient, tightly controlled process; efficient because less than 2.5% of the ALA entering the pathway is lost (Elder, 1982) and tightly controlled because the amount of haem produced closely matches the needs of the body. This implies that enzymes involved in haem synthesis are normally able to use all of the substrate presented to them, that they can handle an increased flux through the pathway, and that the pathway may be subject to some form of “feedback” control. Indeed, there is much evidence to suggest that, at least in the liver and all nonerythroid tissue, haem itself modulates its own rate of production, principally at the level of ALA synthase, which is considered the rate-determining enzyme of the pathway. This tight regulation of liver cell haem occurs by several mechanisms.

First, haem regulates its own synthesis by controlling the amount of ALA-S1 mRNA. In mammalian systems this occurs primarily at the transcriptional level (Srivastava et al, 1988; Yamamoto et al, 1988) but studies in avian systems suggest the effect may also be on mRNA stability (Drew and Ades, 1989; Hamilton et al, 1991). The half-life of mammalian ALA-S1 is less than an hour (Yamamoto et al, 1988) and the half-

life of the protein in mitochondria is even shorter (Kikuchi and Hayashi, 1981). This is an important mechanism as many, but not all drugs, that induce cytochrome P₄₅₀ activity also induce transcription of ALA-S1 in mammalian systems. It is suggested that this may be via haem depletion that would accompany the synthesis of P₄₅₀ haemoprotein.

Secondly, haem regulates the translocation of ALA synthase from cytosol to mitochondria (Yamauchi et al, 1980, Srivastava et al, 1983). This is mediated by two cysteine-containing haem regulatory motifs in the leader sequence (Lathrop and Timko, 1993; Dailey, H. A. – personal communication).

In contrast the mechanisms of controlling erythroid ALA synthase activity are different (Ponka, 1997). Transcription of ALA-S2 is controlled primarily by erythroid-specific transcription factors interacting with noncoding regions of the gene (Cox et al, 1991; May et al, 1995). Interestingly, the same factors are responsible for induction of globin synthesis but this only occurs following the induction of haem synthesis, indicating the importance of haem per se as a regulatory molecule.

Post-transcriptional regulation of ALA-S2 also occurs differently to that of ALA-S1. There is a cis-acting regulatory iron element in the 5' untranslated region (Cox et al, 1991), similar to the stem-loop structure occurring in the 5' untranslated region of ferritin mRNAs (Klausner et al, 1993). A protein that binds to the iron regulatory element inhibits translation of the mRNA, but in the presence of iron the protein dissociates and the mRNA binds to ribosomes and is translated (Melefors et al, 1993). In this way the translation of ALA-S2 mRNA is coupled to the availability of iron.

Although haem does not appear to play a major role in the transcription and translation of ALA-S2, there are identical cysteine-containing haem regulatory motifs to those found in the ALA-S1 gene, in the leader sequence of ALA-S2, and in vitro experiments suggest a haem-mediated inhibitory effect (Lathrop and Timko, 1993). Thus translocation of ALA synthase to the mitochondrion may also be a controlled event in erythroid tissue.

2. ALA Dehydratase [EC 4.2.1.24] (Porphobilinogen Synthase)

The next step in the haem biosynthetic pathway takes place in the cytosol and it involves the condensation of two ALA molecules in a series of stages involving an aldol condensation and formation of a Schiff-base (Shemin, 1976) with the elimination of two water molecules, to form the monopyrrole, porphobilinogen (2nd reaction, figure 1.4) (Jordan and Seehra, 1980). An important role of enzyme catalysis in this reaction is to ensure that the two ALA molecules form the correct product because 2-aminoketones like ALA are intrinsically reactive, and can easily form other products such as the dehydropyrazine formed in the non-enzymatic dimerization. This is the first committed step of the tetrapyrrole synthetic pathway. This reaction is catalysed by the multisubunit cytoplasmic enzyme, ALA dehydratase (Eiberg et al, 1983; Beaumont et al, 1984). Initial evidence for the existence of this enzyme came from studies on ox liver extracts (Gibson et al, 1955) and avian erythrocytes (Schmid and Shemin, 1955).

This protein has been purified from a variety of sources which include yeast (De Barreiro, 1967); *R. sphaeroides* (Nandi et al, 1968); human erythrocytes (Anderson and Desnick, 1979; Gibbs et al, 1985); bovine liver (Bevan et al, 1980; Jordan and Seehra, 1986); and spinach (Liedgens et al, 1983). These enzymes exist as octamers with molecular weights of up to 280 000 Da consisting of identical subunits of approximately 35 000 Da each (Jordan, 1990). The human enzyme is a homo-octamer with subunits of 36 274 Da (Wetmur et al, 1986). The *E. coli* ALA dehydratase was purified and also shown to be a homo-octamer composed of identical 36 554 Da subunits (Spencer and Jordan, 1993). ALA dehydratase of the unicellular green algae, *Scenedesmus obliquus* was identified as a 282 000 Da native protein consisting of six 42 000 Da subunits arranged in a ring (Stolz and Dornemann, 1996).

The genes encoding ALA dehydratases have been sequenced and their cDNAs and protein products characterised from several sources such as human (Wetmur et al, 1986); rat (Bishop et al, 1986); *E. coli* (Echelard et al, 1988); *Chlamydomonas reinhardtii* (Matters and Beale, 1995); soybean (Kaczor et al, 1994); *Bradyrhizobium japonicum* (Chauhan and O'Brian, 1993); pea (*Pisum sativum* L.) (Boese et al, 1991); and *Pseudomonas aeruginosa* (Frankenberg et al, 1998). Two tissue-specific forms of ALA dehydratase exist which are encoded by a single gene which contains separate erythroid and housekeeping promoters and can undergo alternative splicing (Kaya et al, 1994; Bishop et al, 1996). It has been proposed that this novel expression of erythroid-specific and housekeeping transcripts apparently evolved to ensure that there is enough supply of haem for high-level tissue-specific haemoglobin production (Bishop et al, 1996).

In the case of human ALA dehydratase the holoenzyme contains four catalytic sites and can be viewed as a tetramer of dimers with one active site per dimer. Each active site binds two molecules of ALA at two distinct positions, the A-site and P-site (Shoolingin-Jordan et al, 1996, 1997, Shoolingin-Jordan, 1998). The ALA molecule contributing the acetate group and the amino-methyl group of PBG binds at the A-site. The ALA contributing the propionate side chain and the pyrrolic nitrogen binds at the P-site. There is an ordered binding in which the keto group of the ALA contributing the propionate side chain forms a transient covalent bond with a conserved lysine (human Lys-252) in the P-site first. Once there is bound substrate at the P-site with an available 5-amino group binding of the second ALA molecule onto the enzyme at the A-site may occur. The amino-nitrogen is then incorporated into the pyrrole ring of PBG.

Binding of the second substrate at the A-site is dependent on the presence of a divalent metal ion, and removal of these divalent ions prevent binding at the A-site resulting in loss of activity but has no effect on ALA binding at the P-site (Norton et al, 1998). Typically, the required metals are Zn^{2+} or Mg^{2+} (Abdulla and Haeger-Aronsen, 1971, Senior et al, 1996b). Thus in mammalian systems up to a maximum of eight Zn^{2+} ions can bind onto an ALA dehydratase octamer (Wu et al, 1974; Tsukamoto et al, 1980), four being required for catalysis (at the A-site) and four not. Although the binding of the latter four metal ions at the P-site may appear non-essential they probably play a role in conformational stabilisation of the enzyme (Hasnain et al, 1985). The two metal binding sites have been specifically identified on

E. coli ALA dehydratase and are termed the alpha and beta sites (Spencer and Jordan, 1994a; 1995).

The *B japonicum* ALA dehydratase was found to be similar to the plant enzymes in that it required Mg^{2+} ions for activity (Chauhan and O'Brian, 1993). A putative Mg^{2+} binding domain was identified in the cloned *Chlamydomonas reinhardtii* ALA dehydratase, which had approximately 60% amino acid sequence identity to higher plants (Matters and Beale, 1995). Other Mg^{2+} -dependent ALA dehydratases were also identified from *Scenedesmus obliquus*, a unicellular green algae (Stoltz and Dornemann, 1996) and *Pseudomonas aeruginosa* (Frankenberg et al, 1998). Although the *E. coli* ALA dehydratase was shown to be a Zn^{2+} -dependent enzyme, in contrast to the other known prokaryotic ALA dehydratases which are Mg^{2+} -dependent; when a Zn^{2+} ion was present at the beta site, a Mg^{2+} ion could bind at the alpha site and yield an active enzyme (Spencer and Jordan, 1994a, b).

Cloning has allowed the development of expression systems for various forms of ALA dehydratase and ultra-pure preparations of the recombinant enzyme have yielded the protein as a crystal, sometimes suitable for X-ray diffraction (Senior et al, 1996; 1997). The crystallisation and initial X-ray characterisation of the ALA dehydratases from *E. coli* and *Saccharomyces cerevisiae* of around 2Å have been reported (Erskine et al, 1997) as well as a more detailed structure down to 1.67Å for *Pseudomonas aeruginosa* ALA dehydratase (Frankenberg et al, 1999). In all cases the best crystals were obtained when these proteins were covalently bound to laevulinic acid. The X-ray structures have confirmed that ALA dehydratase is a homo-octamer with each of its subunits adopting a "TIM" barrel fold with an N-terminal arm of 30 amino acid residues (Erskine et al, 1999a). The monomers formed asymmetric dimers with their "arms" wrapped around each other, and four of these dimers interact to form octamers with their active sites located on the surface.

In the *E. coli* enzyme Lys-247 (equivalent of the essential Lys-252 at the P-site in human ALA dehydratase) formed a Schiff-base link with the bound laevulinic acid at the active site. This is also the case in the yeast ALA dehydratase where X-ray analysis shows the formation of a Schiff-base with Lys-263 (also equivalent to human Lys-252) (Erskine et al, 1999b).

Pseudomonas aeruginosa ALA dehydratase structural analysis reveals that in each dimer the monomers differed from one another by having a "closed" and an "open" active site pocket (Frankenberg et al, 1999). Whereas no metal ions were found in the active site of both monomers, a single well-defined and highly hydrated Mg^{2+} was identified only in the closed form, about 14Å away from the Schiff base forming nitrogen atom of the active site lysine. Based on this information a structure-based mechanism of action involving Mg^{2+} allosteric binding at the active site and rate enhancement has been proposed (Frankenberg et al, 1999).

Several ALA dehydratase inhibitors have clinical significance. The enzyme is highly sensitive to lead, which is believed to displace the Zn^{2+} ions. The clinical symptoms of lead poisoning are similar to those described in hereditary ALA dehydratase deficiency (table 1.2), suggesting that ALA dehydratase inhibition is responsible for these effects (Granick et al, 1978). Patients with hereditary tyrosinaemia also display

similar symptoms. In these cases a deficiency in the enzyme 4-fumarylacetoacetate hydrolase causes accumulation of succinylacetone, which is a potent inhibitor of ALA dehydratase (Sassa and Kappas, 1983).

3. PBG Deaminase [EC 4.3.1.8] (Hydroxymethylbilane Synthase)

Formation of the basic porphyrinogen tetrapyrrole is initiated in the cytosol by the assembly of four PBG molecules via a stepwise deamination and head-to-tail polymerization into a chemically reactive linear tetrapyrrole, hydroxymethylbilane by the enzyme PBG deaminase (Battersby et al, 1979) (3rd reaction, figure 1.4).

PBG deaminase has been purified from many sources, often as a complex together with the next enzyme in the pathway, uroporphyrinogen-III cosynthase. (Sancovich et al, 1969; Llambias and Batlle, 1970; Frydman and Feinstein, 1974) (see next section). Purification is reported from both eukaryotic and prokaryotic sources such as spinach (Higuchi and Bogorad, 1975); human erythrocytes (Anderson and Desnick, 1980; Corrigan et al, 1991); *Rhodobacter spheroides* (Jordan and Shemin, 1973; Davies and Neuberger, 1973); and *E. coli* (Hart and Battersby, 1985). The purified protein exists as a 35 000 to 44 000 Da monomer, with optimal activities at pH 8.0-8.5 (Jordan, 1990).

PBG deaminase is unique in that it contains a covalently attached dipyrromethane cofactor at the active site which binds substrate molecules during the sequential assembly of the linear tetrapyrrole molecule (Jordan and Warren, 1987). The structure of the dipyrromethane cofactor and its sites of attachment to the enzyme have been characterised (Jordan et al, 1988; Jordan, 1990). The PBG deaminase apoenzyme catalyses the deamination and polymerisation of two molecules of PBG at its active site (Awan et al, 1997). The resultant dipyrrole is covalently linked via a thioether linkage to the enzyme through a conserved cysteine (*E. coli* Cys-242) (Jordan et al, 1988; Louie et al, 1996). This dipyrrolic cofactor then acts as a primer which gets elongated in a stepwise mechanism, one PBG unit at a time, through enzyme intermediate complexes, ES (with one PBG attached); ES2 (two PBGs attached); ES3 (three PBGs attached), and finally ES4 (four PBGs attached) from which the tetrapyrrole product, hydroxymethylbilane, is released by hydrolytic cleavage, regenerating the enzyme-dipyrromethane intact (Jordan, 1994; Luke and Shoolingin-Jordan, 1998). Thus, the two proximal PBGs (ie. the dipyrromethane cofactor) remain covalently linked to the enzyme and are not turned over. The precise mechanisms by which the enzyme carries out this sequential manipulation of the four substrates and how the tetrapyrrole product is specifically cleaved leaving the intact dipyrromethane cofactor covalently attached to the enzyme, have not been fully elucidated.

PBG deaminase was the first of the haem biosynthetic enzymes to benefit from the application of modern recombinant DNA technological investigation (Grandchamp et al, 1984). Presumably this was primarily driven by investigators attempting to derive diagnostic benefit in the realm of the clinically important, diagnosis of acute intermittent porphyria (see below). Consequently the gene for PBG deaminase has been sequenced and characterised from both prokaryotic and eukaryotic sources (Grandchamp et al, 1984; Thomas and Jordan, 1986; Sasarman et al, 1987; Jordan et al, 1987; Astrin et al, 1991; Cardalda et al, 1998). Comparison of the deduced primary

protein structures of PBG deaminases from various sources reveal a considerable degree of conservation of this enzyme during evolution, as demonstrated by the 60% similarity between the human and the *E. coli* enzymes (Shoolingin-Jordan, 1998).

In mammals there is a single PBG deaminase gene consisting of 15 exons extending over 10kb of DNA (Deybach and Puy, 1995). In humans the gene has been mapped to chromosome 11q23 and two different transcripts, differing at their 5' ends, are produced from the single gene (Namba et al, 1991, Deybach and Puy, 1995). The first is a ubiquitous, "housekeeping" mRNA transcript produced in all cells in which exon 1 is spliced to exon 3. The second form is specific to erythroid cells and initiates by alternate splicing at exon 2 (Grandchamp et al, 1987). Exon 2 does not contain an AUG translation-initiating codon, and translation of the erythroid-specific mRNA is initiated at an AUG located in exon 3 (Chretien et al, 1988). Exon 1 contains an AUG, which is spliced into the same reading frame as the AUG in exon 3. Thus, translation of the "housekeeping" mRNA produces a protein that differs from the erythroid form by the absence of 17 amino acids (encoded by exon 1) at its N-terminus (Grandchamp et al, 1987).

Activation of transcription of PBG deaminase is controlled by two separate promoters. The "housekeeping" promoter lies upstream of exon 1 and contains motifs commonly associated with "housekeeping" genes (Grandchamp et al, 1987, Chretien et al, 1988). The erythroid promoter lies between exon 1 and 2. The two promoters appear to be regulated independently, even though when RNA polymerase initiates at the "housekeeping" promoter it must transcribe the entire erythroid promoter (Chretien et al, 1988). The erythroid-specific promoter contains at least three classes of transcription factor binding sites characteristic of erythroid promoters, viz. the GATA, NF-E2 and the CACCC motifs (Mignotte et al, 1989). In keeping with other erythroid promoters, activation of transcription requires an interaction between the factors bound at the GATA site and factors bound at either NF-E2 or the CACCC sites (Mignotte et al, 1989).

In addition to the cloning and large scale expression of recombinant *E. coli* PBG deaminase (Jordan et al, 1988; 1992) allowing characterisation of the dipyrromethane cofactor, crystallisation of the protein was facilitated and the X-ray crystal structure of the *E. coli* PBG deaminase determined to 1.76Å resolution (Louie et al, 1992; 1996). The high resolution structure revealed a protein folded into three alpha/beta domains of approximately 100 amino acids each, linked to one another by flexible strands (Lambert et al, 1994; Louie et al, 1996; Shoolingin-Jordan, 1998; Luke and Shoolingin-Jordan, 1998). Domains 1 and 2, which have similar overall topology form a cleft at their interface. The dipyrromethane cofactor is bound by extensive contacts, including salt bridges and hydrogen bonds between these two domains in this cleft. Site-directed mutagenesis experiments have demonstrated that several of the salt bridges between arginine and the pyrrole acetates and propionates are important for enzymatic activity (Lambert et al, 1994, Jordan, 1994). Domain 3, which is an open-faced anti-parallel sheet of three strands containing the cysteine to which the cofactor is covalently bound, is situated deep within the cleft between domains 1 and 2. Deamination of PBG and formation of the methene bridge occurs here. Thus domain 3 can be considered as containing the single catalytic site. Importantly, the crystal structure shows flexible boundaries between the 3 domains,

which would allow conformational changes that accommodate each added PBG pyrrole until the tetrapyrrole (hydroxymethylbilane) is synthesised.

4. Uroporphyrinogen Cosynthase [EC 4.2.1.75]

The regularly substituted linear tetramer, hydroxymethylbilane, is converted to an asymmetrically substituted cyclic tetramer, uroporphyrinogen-III, by uroporphyrinogen cosynthase (4th reaction, figure 1.4). In the absence of this enzyme hydroxymethylbilane cyclizes spontaneously to form uroporphyrinogen-I instead of the III isomer which, as noted above, is required for further processing to haem (Jordan, 1994). Thus cyclization of hydroxymethylbilane by uroporphyrinogen cosynthase critically involves intramolecular inversion of the terminal D ring of the substrate. This inversion probably occurs via a chiral spiro intermediate (Leeper, 1994; Spivey et al, 1996). Considering the requirement for uroporphyrinogen cosynthase to act in concert with its "partner", PBG deaminase, and the fact that the two enzymes may be co-purified, it has been suggested that they may exist in a cytosolic complex (Tsai et al, 1987).

Uroporphyrinogen cosynthase has been isolated and purified to homogeneity from many sources (Higuchi and Bogorad, 1975; Kohashi et al, 1984; Smythe and Williams, 1988; Hart and Battersby, 1985; Alwan and Jordan, 1988) including human erythrocytes (Tsai et al, 1987). They all appear to exist as monomeric subunits with molecular weights around 30 000 Da (Jordan, 1990) and are extremely thermolabile. Because of this instability the protein has not been well characterised. There is no evidence for a cofactor of any sort and the human enzyme has an isoelectric point of 5.5, and a pH optimum of 7.4. Activity measurements show it to be present in excess over PBG deaminase, favouring the synthesis of the uroporphyrinogen-III over the series I isomeric form (Tsai et al, 1987, Desnick et al, 1998).

The genes/cDNAs encoding this enzyme have been isolated from several sources including animals and bacteria (Stamford et al, 1995; Amillet and Labbe-Bois, 1995; Xu et al, 1995; Tsai et al, 1988; Jordan et al, 1988). The human uroporphyrinogen cosynthase gene has been isolated, sequenced and mapped to chromosome 10q25.3 (Astrin et al, 1991), and the cDNA expressed in *E. coli* (Tsai et al, 1988). In both the human and mouse there are 5' and 3' untranslated regions and an open reading frame spanning 10 exons and encoding a polypeptide of 265 amino acids. The mouse gene shares an 80% nucleotide and 78% amino acid identity with that of the human gene (Xu et al, 1995).

5. Uroporphyrinogen Decarboxylase [EC 4.1.1.37]

Uroporphyrinogen decarboxylase, a soluble cytoplasmic protein, catalyses the decarboxylation of four acetyl side-chains in the 8-carboxylic (-COOH) uroporphyrinogen-III molecule. The reaction proceeds in a stepwise manner through formation of 7-, 6-, and 5-COOH intermediates resulting in the formation of the 4-COOH coproporphyrinogen-III (Mauzerall and Granick, 1958; Jackson et al, 1976) (5th reaction, figure 1.4).

In theory, the successive removal of all four acetate carboxyl groups could occur by 24 possible routes involving fourteen intermediates (McDonagh and Bissell, 1998). Nevertheless evidence suggests that, at physiologic substrate concentrations, this reaction occurs in an orderly manner with the carboxyl groups removed in a clockwise direction starting at ring D and proceeding through A, B, and C before the final formation of coproporphyrinogen-III (Jackson et al, 1976; Luo and Lim, 1993). The intermediates formed in this reaction are relatively stable porphyrinogen species which are detectable *in vivo*. Each intermediate acts as the substrate for further decarboxylation until the requisite coproporphyrinogen-III is formed. Both the series I and III uroporphyrinogen isomers formed are suitable substrates for this enzyme, but the series III isomer is more rapidly decarboxylated (Granick and Mauzerall, 1958; Smith and Francis, 1981).

This enzyme has been purified from human erythrocytes (de Verneuil et al, 1983; Elder et al, 1983; Mukerji and Pimstone, 1992; Roberts and Elder, 1997); bovine liver (Straka and Kushner, 1983); chicken erythrocytes (Kawanishi et al, 1983; Seki et al, 1986); *Euglena gracilis* (Juknat et al, 1989); *Saccharomyces cerevisiae* (Felix and Brouillet, 1990) and *Rhodobacter sphaeroides* (Jones and Jordan, 1993). Most forms of the protein have been reported to be monomeric with molecular weights reported from 40 000 Da (Elder et al, 1983) to 46 000 Da (de Verneuil et al, 1983). The chicken enzyme, on the other hand is reported to exist as a dimer composed of two 40 000 Da subunits (Kawanishi et al, 1983).

The cDNAs for the gene encoding this protein in humans and rat have been cloned, sequenced and characterised (Romeo et al, 1986; Romana et al, 1987a). The two uroporphyrinogen decarboxylase gene sequences are very similar with 85% and 90% homology at the DNA and protein levels, respectively. The cDNA for the mouse gene encoding this enzyme has 88% and 90% nucleotide and amino acid sequence identity, respectively, with the human enzyme (Wu et al, 1996). The isolated human uroporphyrinogen decarboxylase gene is made up of 10 exons and two transcriptional start sites were identified (Romana et al, 1987b). The human gene encodes a 367 amino acid residues polypeptide (predicted molecular weight approximately 41 000 Da), is present as a single copy containing 10 exons within about 3 kb of DNA and has been mapped to chromosome 1p34 (de Vernueil et al, 1983; Dubart et al, 1986; Romeo et al 1986). Two transcriptional start sites separated by six nucleotides have been identified (Romana et al, 1987b) but the same polyadenylation site is used in all tissues and it seems that both sites are used in the same proportion in both erythroid and nonerythroid cells. Two isoforms of this enzyme were identified in human erythrocytes by Murkeji and Pimstone (1987).

cDNAs from other species have also been analysed and compared. Sequence analysis of *S. cerevisiae* (Garey et al, 1992; Difflumeri et al, 1993) and *Synechococcus* (Kiel et al, 1992) uroporphyrinogen decarboxylase revealed approximately 50% and 32% respectively identity with the human enzyme. In addition a full length cDNA for the tobacco plant (*Nicotiana tabacum* L.) uroporphyrinogen decarboxylase gene and a partial cDNA for the barley (*Hordeum vulgare* L.) gene have been cloned and sequenced (Mock et al, 1995). Overall, in terms of protein size, amino acid composition, kinetic properties and physicochemical characteristics uroporphyrinogen

decarboxylases compared from all these different sources (bacteria, yeast, plants, mammals) are fundamentally similar (Elder and Roberts, 1995).

Various inhibitor studies using human uroporphyrinogen decarboxylase suggest that cysteine and histidine residues are important for enzyme activity especially free thiols and various conserved histidines, lysines and arginines (Whitby et al, 1998). However, site-directed mutagenesis experiments indicate that no single cysteine is absolutely critical for the integrity of the catalytic site, as activity in all cysteine mutants was essentially maintained at a significant level. Three histidine mutants also retained significant enzyme activity but one (human H339N) has been identified as important in imparting isomer specificity (Wyckoff et al, 1996).

A recombinant human uroporphyrinogen decarboxylase, expressed with a histidine tag in *E. coli* for purification purposes, was crystallized and initial data collected at a 3.0Å resolution (Phillips et al, 1997; Laterriere et al, 1997). Subsequently, the crystal structure has been determined at 1.60Å resolution (Whitby et al, 1998). This enzyme is a dimer in solution and this dimer also appears to be formed in the crystal (Whitby et al, 1998).

6. Coproporphyrinogen Oxidase [EC 1.3.3.3]

At this point the synthesis of haem re-enters the mitochondria, where the enzyme coproporphyrinogen-III oxidase, located in the intermembrane space and loosely associated with the inner mitochondrial membrane on the intermembrane space side (Sano and Granick, 1961; Battie et al, 1965; Poulson and Polglase, 1974; Elder and Evans, 1978; Grandchamp et al, 1978), catalyses the oxidative decarboxylation of coproporphyrinogen-III to form protoporphyrinogen-IX (6th reaction, figure 1.4).

The two propionate residues (P) on rings A and B of the tetrapyrrole molecule are converted into vinyl groups ($-\text{CH}=\text{CH}_2$) in a clockwise fashion, proceeding via a tricarboxylic porphyrinogen, tripropionate monovinyl porphyrinogen (trivially known as harderoporphyrinogen). It is a stepwise decarboxylation with the decarboxylation of the position 2 propionate side chain proceeding first and at a faster rate than that of position 4 (Martasek, 1998). Under normal circumstances free harderoporphyrinogen is not generated and thus does not appear in physiological porphyrin analytical profiles. Two molecules of CO_2 are produced and the reaction requires molecular oxygen as an electron acceptor. This oxygen-dependent reaction probably involves β -hydroxypropionate which is then decarboxylated and at least one protein tyrosine group(s) may be involved in this part of the catalytic mechanism (Yoshinaga and Sano, 1980b). In an alternative scheme it has been suggested that a hydroxylation reaction does not take place, but the reaction proceeds via a hydride removal during the decarboxylation (Seehra et al, 1982). It is possible that both schemes may occur naturally depending on the nature of the organism (Dailey, 1990). Indeed, in prokaryotes there appears to be two forms of coproporphyrinogen oxidase based on their oxygen requirements, and in the case of *Salmonella typhimurium* two genes, encoding an oxygen-dependent and oxygen-independent coproporphyrinogen-III oxidase, have been cloned and sequenced (Xu and Elliot, 1993, 1994).

Coproporphyrinogen oxidase has been purified from a number of sources including rat (Batlle et al, 1965), bovine (Yoshinaga and Sano, 1980a; Kohno et al, 1993) and mouse liver (Bogard et al, 1989) and *Saccharomyces cerevisiae* (Camadro et al, 1986). Early studies reported molecular weights in the region of 70 – 80 000 Da (Batlle et al, 1965; Yoshinaga and Sano, 1980a), and later studies indicate a dimeric protein consisting of approximately 35 000 Da molecular weight subunits (Camadro et al, 1986; Bogard et al, 1989; Kohno et al, 1993). cDNA data has confirmed this.

The cDNA sequence of the human gene encoding coproporphyrinogen-III oxidase has been cloned, sequenced and characterised (Taketani et al, 1994; Martasek et al, 1994; Delfau-Larue et al, 1994; Medlock and Dailey, 1996). Analysis of the cDNA and the cloned gene from a number of oxygen-dependent species as well as the N-terminal amino acid sequence from mouse liver has led workers to suggest that newly synthesised coproporphyrinogen-III oxidase has an N-terminal mitochondrial-targeting peptide which is cleaved during transport into the mitochondria. Although the length of this leader sequence was initially proposed to be 31 amino acid residues in length (Kohno et al, 1993, Taketani et al, 1994) other workers have reported an unusually long leader sequence of 110 amino acids (Delfau-Larue et al, 1994, Martasek et al, 1994).

In humans there appears to be a single copy of the gene with multiple transcriptional initiation sites. The human gene spans approximately 14 kilobases, and consists of seven exons and six introns (Delfau-Larue et al, 1994). This gene has been mapped to chromosome 3q12 of the human genome (Cacheux et al, 1994). Potential regulatory elements have been identified in the GC-rich promoter region (six Sp1, one CACCC and four GATA sites) and it is suggested that a single promoter may be differentially regulated in erythroid and non-erythroid tissue (Martasek et al, 1994, Taketani et al, 1994). Coproporphyrinogen-III oxidase transcripts are induced during erythroid cell differentiation (Conder et al, 1991, Taketani et al, 1995a). The gene contains two polyadenylation signals separated by 126 base pairs and it is possible that these two signals, differently utilised, may play a role in tissue-specific expression of coproporphyrinogen-III oxidase mRNA (Martasek et al, 1997).

Kohno et al (1993) isolated the full length cDNA sequence of the mouse coproporphyrinogen oxidase gene from a mouse erythroleukemia cell cDNA library. Sequence analysis revealed that the mouse gene encodes a 40 647 Da (354 amino acid residues) protein precursor with a putative cleavable leader peptide of 31 amino acid residues, which was modified into a 37 255 Da (323 amino acid residues) mature protein. The post-translational modification of the mouse enzyme was very similar to the human form. Localisation experiments revealed that the enzyme activity was mainly found in mitochondria. Metal analysis and site-directed mutagenesis experiments indicated that the mouse enzyme was a metalloprotein associated with Cu^{2+} ions as essential cofactors for the enzyme activity (Kohno et al, 1996).

The cDNA encoding human coproporphyrinogen oxidase gene was expressed in *E. coli* cells and purified to apparent homogeneity (Medlock and Dailey, 1996). Metal analysis of this purified protein by u.v./visible spectroscopy, inductively coupled plasma atomic emission spectroscopy, and electron paramagnetic resonance

spectroscopy revealed that the human enzyme did not have a metal centre and there was no *in vitro* stimulation by either Fe^{2+} or Cu^2 .

In prokaryotes there appears to be two forms of coproporphyrinogen oxidase, based on their oxygen requirements. The genes for these coproporphyrinogen oxidase forms viz., *hemF* and *hemN* code for the oxygen-dependent and oxygen-independent enzymes, respectively. Xu and Elliot (1993) reported the cloning and sequencing of the *hemF* gene which encodes an oxygen-dependent coproporphyrinogen oxidase from the facultative bacterium, *Salmonella typhimurium*. The predicted protein sequence had 44% amino acid identity to the yeast coproporphyrinogen oxidase encoded by *hem13* gene (Zagorec et al, 1988). Subsequently, these workers cloned and sequenced the *hemN* gene which encodes the oxygen-independent enzyme (52 800 Da) from the same organism (*S. typhimurium*) (Xu and Elliot, 1994). This protein had 38% identity to a putative anaerobic coproporphyrinogen oxidase (*hemN*) of *Rhodobacter sphaeroides* which had previously been cloned and sequenced (Coomber et al, 1992). The two genes encoding the aerobic (*hemF*) and anaerobic (*hemN*) coproporphyrinogen oxidase isoforms of *E. coli* were also cloned and characterised (Troup et al, 1994; 1995). An open reading frame (*hemN*) cloned from *B. subtilis* was also identified to be coding for an oxygen-independent coproporphyrinogen-III decarboxylating protein (Hippler et al, 1997). Detailed investigation for the *hemF* (oxygen-dependent) gene in the *B. subtilis* genomic DNA, and alignment with all known sequences, could not identify this gene in this organism (Hippler et al, 1997).

Zagorec et al (1988) isolated the *hem13* gene of *Saccharomyces cerevisiae* by functional complementation of a *hem13* mutant. The encoded protein had a calculated molecular weight of 37 673 Da (328 amino acid residues) which was in agreement with that predicted for the previously purified aerobic native enzyme (Camadro et al, 1986). The yeast coproporphyrinogen oxidase is different from others in that, it exists as a cytoplasmic rather than a mitochondrial protein. The cDNA for the gene encoding coproporphyrinogen oxidase of soybean plant was isolated and its primary structure was determined (Madsen et al, 1993). The gene encodes a polypeptide with a predicted molecular weight of 43 000 Da which has 50% amino acid sequence similarity to the yeast coproporphyrinogen oxidase. A 67 amino acid residue extension at the N-terminus of the protein, which is different to the other coproporphyrinogen oxidase sequence, is thought to be a leader peptide required for transit across membranes. This protein was shown to be highly expressed in soybean root nodules.

7. Protoporphyrinogen Oxidase (PPO) [EC 1.3.3.4]

In the penultimate step of the haem biosynthesis pathway protoporphyrinogen-IX is oxidised to protoporphyrin-IX by protoporphyrinogen oxidase (7th reaction, figure 1.4).

This was the last enzymatic reaction to be described in haem biosynthesis and is the least characterised. Initial evidence which indicated the enzymatic oxidation of protoporphyrinogen-IX first emerged when Sano and Granick (1961) demonstrated the *in vitro* increase in the rate of this reaction on addition of liver cell mitochondrial extract. A similar effect was observed on addition of bovine liver mitochondrial

extracts (Porra and Falk, 1961). Subsequent studies showed that oxygen was the acceptor of the electrons which were released during the oxidation of protoporphyrinogen-IX to form protoporphyrin-IX (Porra and Falk, 1964).

Important breakthroughs in describing the enzymatic catalysis of this reaction came from the work done by Poulson and Polglase (1975), and Poulson (1976) which provided much of the evidence which showed that a protein associated with mitochondrial membranes was involved in this reaction. The detailed localisation studies done by Deybach et al (1985) confirmed the mitochondrial location of this protein and further provided evidence showing that this enzyme was intrinsically associated with the inner mitochondrial membrane. The work by Ferreira et al (1988) further confirmed these findings and they proposed that the terminal three enzymes of the haem biosynthesis pathway that is, coproporphyrinogen oxidase; protoporphyrinogen oxidase; and ferrochelatase, could possibly exist in some form of an enzyme complex which would facilitate the channelling of substrate intermediates between the different enzymes. Thus far the existence of such a complex has not been verified.

In the next few of years great effort was put into trying to characterise this protein from various sources. For many years scientists were dogged by difficulties in trying to purify the protein from membranes using conventional chromatographic techniques. PPOs are hydrophobic membrane bound proteins which require detergents for solubilisation. Repeated purification attempts in different laboratories around the world resulted in partial characterisation of the enzyme from several sources such as rat (Poulson, 1976); *Saccharomyces cerevisiae* (Poulson and Polglase, 1975; Camadro et al, 1994); *Rhodopseudomonas spheroides* (Jacobs and Jacobs, 1981) barley (Jacobs and Jacobs, 1987; Jacobs et al, 1989); *Desulfovibrio gigas* (Klemm and Barton, 1987); mouse (Dailey and Karr, 1987; Ferreira and Dailey, 1988; Proulx and Dailey, 1992) and bovine (Siepker et al, 1987).

More detail and progress made in characterising PPOs is presented separately, later in this review.

8. Ferrochelatase (FC) [EC 4.99.1.1]

The last step in haem biosynthesis involves the incorporation of ferrous iron (Fe^{2+}) into the protoporphyrin-IX macrocycle by ferrochelatase to form haem (protohaem) (Goldberg et al, 1956) (8th reaction, figure 1.4). This reaction has been extensively studied and is well characterised in humans. Ferrochelatase is an intrinsic protein of the inner mitochondrial membrane (McKay et al, 1969; Jones and Jones, 1969; Harbin and Dailey, 1985). Initial attempts at purifying this protein to homogeneity were hampered by difficulties in stabilizing the enzyme during the purification process. Relentless efforts by various laboratories resulted in the purification of the protein from various sources such as rat liver (Mailer et al, 1980; Taketani and Tokunaga, 1981), *Rhodopseudomonas sphaeroides* (Dailey, 1982), bovine liver mitochondria (Taketani and Tokunaga, 1982; Dailey and Fleming, 1983; Siepker et al, 1987). The bovine enzyme with a reported molecular weight of 40 000 Da was purified and shown to be cross-reacting with antibodies raised against the purified bovine PPO (Siepker et al, 1987) although this has never been confirmed by others.

The structural gene for *Saccharomyces cerevisiae* ferrochelatase (*hem15*) was isolated by functional complementation of a mutant yeast strain (Labbe-Bois, 1990). This gene encodes a protein precursor with a putative 31 amino acid residues leader sequence and the mature enzyme is a 40 900 Da (362 amino acid residues) protein. Eldridge and Dailey (1992) described the production of large amounts of the yeast ferrochelatase from a baculovirus expression system. Investigation of several invariant amino acid residues in the yeast ferrochelatase protein by site-directed mutagenesis revealed that Glu-314 was critical for catalysis, for metal binding His-235 was essential, and Asp-246 and Tyr-248 were also involved synergistically in this function (Gora et al, 1996).

Mouse ferrochelatase gene cDNA was isolated by immunoscreening of a cDNA library from mouse erythroleukemia (MEL) cells with a polyclonal antibody (Taketani et al, 1990). This gene encodes a ferrochelatase precursor of 47 130 Da (420 amino acid residues) with a putative presequence of 53 amino acid residues which on cleavage during importation into the mitochondrial inner membrane is modified into a mature protein of 41 692 Da (367 amino acid residues). Brenner and Frasier (1991) also reported the isolation of a mouse ferrochelatase gene from libraries screened with an oligonucleotide probe, and found similar features to those reported by Taketani et al (1990). The deduced amino acid sequence had 47% identity with the cloned yeast ferrochelatase. A single chromosomal gene was demonstrated by Southern blotting, whilst Northern blotting showed existence of two transcripts in all tissues (Brenner and Frasier, 1991). The cDNA of the gene encoding mouse ferrochelatase was overexpressed in *E. coli* and this facilitated the purification of the enzyme, but no further characterisation was performed (Ferreira, 1994). Purification of large amounts of the mammalian ferrochelatase and characterisation was reported by Dailey et al (1994b).

The characterisation of the mouse ferrochelatase gene facilitated the cloning and sequencing of the human gene (Nakahashi et al, 1990). This human ferrochelatase gene was isolated from a placental cDNA library using a radiolabelled mouse cDNA fragment. This enzyme was synthesized as a 47 833 Da (423 amino acid residues) protein precursor which was modified into a smaller mature protein of 42 158 Da by cleavage of a putative leader sequence of 54 amino acid residues. Analysis of the primary sequence of this protein showed identities of 88% and 46% to the mouse and yeast ferrochelatases, respectively. Human ferrochelatase gene was mapped to chromosome 18q22 by hybridisation of the cDNA to sorted chromosomes and by fluorescence *in situ* hybridisation with a human ferrochelatase genomic clone (Whitcombe et al, 1991; Taketani et al, 1992, Brenner et al, 1992). The cloned mature forms of the human and mouse ferrochelatases were expressed, purified and characterised (Dailey et al, 1994b, c). In this study this group demonstrated the intriguing presence of a single labile iron-sulphur (2Fe-2S) cluster at the carboxyl end of the purified mature human ferrochelatase using uv/visible spectral analysis, variable temperature magnetic circular dichroism (MCD) and electron paramagnetic resonance (EPR). The labile [2Fe-2S] cluster was shown to be disappearing gradually over 24 hours, with concomitant loss of enzyme activity when stored aerobically at 4°C. Investigation of the purified mouse natural and recombinant ferrochelatases using EPR and Mossbauer spectroscopy also confirmed the presence of the [2Fe-2S] cluster in these enzymes (Ferreira et al, 1994). In the same study, sequence analysis of

the prokaryotic, yeast, and plant ferrochelatase gene cDNAs revealed a lack of the putative binding site for the iron sulphur cluster. The destruction or loss of this cluster from the mammalian ferrochelatases resulted in loss of enzyme catalytic activity (Sellers et al, 1996). In that study EPR and uv/visible spectroscopy was applied to demonstrate the destruction of the [2Fe-2S] cluster by nitric oxide (NO) in both the human and mouse ferrochelatases. Interestingly, NO had no effect on the yeast ferrochelatase which lacks the [2Fe-2S] cluster, or other enzymes such as spinach ferredoxin which also has the [2Fe-2S] cluster. Further investigations involving site-directed mutagenesis and spectroscopic characterisation of the human enzyme revealed that three cysteines at the carboxyl terminal; Cys-403, Cys-406 and Cys-411 (which are conserved in many of the known ferrochelatase sequences) were involved in the ligation of the [2Fe-2S] cluster to the protein (Crouse et al, 1996). Another cysteine, Cys-196, has been identified as the fourth ligand of the [2Fe-2S] onto human ferrochelatase (Sellers et al, 1998). The precise function of the iron-sulphur centre remains to be elucidated. But since this centre appears to be absent in bacterial and yeast ferrochelatases, it was proposed that this [2Fe-2S] centre may be involved in a regulatory role in the mammalian enzymes (Sellers et al, 1998).

Recent studies have revealed the presence of the [2Fe-2S] cluster in several non-mammalian ferrochelatases (Sellers et al, 1998; Day et al, 1998). EPR and uv/visible spectroscopy were applied in identifying the [2Fe-2S] cluster in the cloned *Drosophila melanogaster* ferrochelatase (Sellers et al, 1998). The conserved cysteines 196, 403, 406 and 411 were also identified in this enzyme. Day et al (1998) reported the cloning of the chicken (*Gallus gallus*) and amphibian (*Xenopus laevis*) ferrochelatases, and the expressed (in *E. coli*) and purified proteins were both found to contain [2Fe-2S] clusters which were readily destroyed by NO.

The gene for a prokaryotic ferrochelatase was identified and characterised from the aerobic bacterium *Bacillus subtilis* (Hansson and Hederstedt, 1994a). In this organism this gene forms part of the *hemEHY* gene cluster which encodes uroporphyrinogen (*hemE*), protoporphyrinogen oxidase (*hemY*) and ferrochelatase (*hemH*) (Hansson and Hederstedt, 1992, 1994b). The purified protein encoded by *hemH* was shown to be a water-soluble, 35 000 Da monomeric protein which could incorporate Fe²⁺, Zn²⁺ and Cu²⁺ into the protoporphyrin-IX macrocycle. A single cysteine residue appeared to be required for catalytic activity of this enzyme. Hansson and Al-Karadaghi (1995) expressed the *B. subtilis* ferrochelatase as a soluble protein in *E. coli* cells and purified it for crystallisation studies. Subsequently, the three dimensional crystal structure of this protein was determined at 1.9Å resolution by a method of multiple isomorphous replacement, and characterised (Al-Karadaghi et al, 1997). This protein was folded into two similar domains each with a four-stranded parallel beta sheet flanked by alpha helices. The two domains formed a cleft with several of the amino acids which are invariant in many ferrochelatase species. It was proposed that the porphyrin substrate binds in this cleft and that the metal-binding site was also found here (Al-Karadaghi et al, 1997).

The Porphyrrias

The porphyrias are a heterogeneous group of metabolic disorders associated with specific clinical syndromes. They are characterised by a derangement of the haem biosynthetic pathway resulting from inherited deficiencies of the enzymes catalysing the reactions in this biochemical pathway (Kappas et al, 1989). Thus, each of the different porphyrias is characterised by a defect in a specific haem synthetic enzyme (table 1.1).

Table 1.1 A summary of the porphyria syndromes and the haem synthetic enzymes affected in each of these conditions.

Enzyme involved	Haem synthetic pathway	Porphyria syndrome
	Glycine + Succinyl CoA	
ALA synthase	↓	
	5-aminolaevulinic acid	
ALA dehydratase	↓	ALA dehydratase deficiency
	Porphobilinogen	
Porphobilinogen deaminase	↓	Acute intermittent porphyria
	Hydroxymethylbilane	
Uroporphyrinogen cosynthase	↓	Congenital erythropoietic porphyria
	Uroporphyrinogen-III	
Uroporphyrinogen decarboxylase	↓	Porphyria cutanea tarda
	Coproporphyrinogen-III	
Coproporphyrinogen oxidase	↓	Hereditary coproporphyria
	Protoporphyrinogen-IX	
Protoporphyrinogen oxidase	↓	Variegate porphyria
	Protoporphyrin-IX	
Ferrochelatase	↓	Erythropoietic porphyria
	Haem	

The defect is often identifiable as decreased catalytic activity in that enzyme (Waldenstrom, 1957; Brodie et al, 1977b; Elder, 1982; Rimington, 1985). The focus of this dissertation, PPO, is associated with variegate porphyria (VP). In rare circumstances more than one enzyme may be affected (Day et al, 1982; Martasek et al, 1983; Sturrock et al, 1989). Deficiencies in the enzyme activities are either due to defective protein structure, or reduced synthesis of the normal protein (Moore et al,

1987). At the biochemical level, the type of porphyria is identified by the characteristic excretion pattern of porphyrin intermediates present in urine, stool and blood from patients, which reflects the enzyme deficiency, and this is sometimes accompanied by an elevation of the porphyrin precursors, 5-aminolaevulinic acid (ALA) and porphobilinogen (PBG) in the acute porphyrias (Moore et al, 1987; Dailey, 1990; Kappas et al, 1989).

The inherited porphyrias appear to be of variable clinical penetrance. In some studies only about 10% of subjects who carry a particular defective enzyme develop clinical symptoms or other biochemical changes associated with the disorder (Moore et al, 1987; Hindmarsh, 1993; Elder, 1993; Elder et al, 1997). In other studies the penetrance may be in the range of 40 – 60 % (Kauppinen and Mustajoki, 1992). Generally, the prevalence of overt cases of the porphyrias is much lower than the defective gene frequencies.

Porphyrias have a worldwide distribution, but certain types have exceptionally high frequencies in some countries. This is demonstrated by the relatively high frequency of VP in the South African population which has been attributed to a founder gene effect (Dean, 1971; Meissner et al, 1987, 1996). More recent work has established a molecular basis for this. The gene defect apparently responsible for the VP founder effect in South Africa, is termed R59W and was found in over 90% of VP cases that were studied (Meissner et al, 1996). This is reviewed in more detail later. Similarly in Northern Sweden a single mutation termed Trp198Stop, which is presumably responsible for a founder effect of acute intermittent porphyria (AIP) in that country appears to be responsible for the high prevalence (1/1500 people) of this disease in that area of that country (Kauppinen and Mustajoki, 1992).

Classification of Porphyrias

The diseases of porphyrin metabolism were first classified by Gunther (1911; 1922). Several classifications followed and the general aim of these was to provide a clinical basis that was consistent with the biochemical changes. A useful classification of the porphyrias groups these disorders into acute and non-acute forms on the basis of their main clinical presentation. Such a classification takes into account the combination of clinical manifestations and biochemical changes associated with these disorders and allows for differential diagnosis of the different porphyrias (Moore, 1990; Meissner, 1990). The clinical disease state of porphyrias involves several different body systems which may include the skin, nervous system, digestive system, and liver, depending on the type and level of expression of porphyria (Brodie et al, 1977a; Elder, 1982; Rimington, 1985). As it is not the purpose of this dissertation to discuss all the porphyrias in detail I have included table 1.2 which classifies the porphyrias as acute or non-acute and includes a certain amount of detail in summary form.

Table 1.2: A classification of the porphyrias.

5-ALA-D deficiency = 5-aminolaevulinic acid dehydratase deficiency porphyria;

AIP = Acute intermittent porphyria; CEP = Congenital erythropoietic porphyria;

PCT = Porphyria cutanea tarda; HCP = Hereditary coproporphyria;

VP = Variegate porphyria; EP = Erythropoietic protoporphyria.

Porphyric syndrome	Affected enzyme	Inheritance pattern	Chromosomal location
The Acute Porphyrias			
5-ALA-D deficiency	5-ALA dehydratase	Recessive	9q34
<p>Comment: ALA dehydratase porphyria results from a homozygous 5-ALA deficiency, and heterozygotes with a genetic defect usually do not show clinical changes (Sassa, 1998). Diminished 5-ALA dehydratase activity was demonstrated in erythrocytes (Doss et al, 1982). It is the rarest form of porphyria (Kappas et al, 1995). Disease presents prepubertally. Is similar to lead poisoning, but lead poisoning is generally associated with elevated urinary ALA and coproporphyrin. Symptoms similar to AIP, but can be differentiated by its autosomal recessive inheritance, lack of PBG overproduction and markedly reduced ALA dehydratase activity (Sassa, 1998). No cutaneous photosensitivity. Molecular analysis of the ALA dehydratase genetic defects revealed different mutations indicating the genetic heterogeneity of this condition (Ishida et al, 1990; Plewinska et al, 1991;). Patients with ALA dehydratase genetic defects showed markedly elevated urinary ALA, but variation of the clinical disease ranging from asymptomatic to a severe porphyric syndrome (Sassa, 1998).</p>			
AIP	PBG Deaminase	Dominant	11q24.1-24.2
<p>Comment: Autosomal dominant disorder in which the activity of PBG deaminase is reduced in circulating erythrocytes (Strand et al, 1972) and other cells such as liver (Miyagi, 1970), lymphocytes (Sassa et al, 1978), fibroblasts (Meyer, 1973), and amniotic cells (Sassa et al, 1975). Enzyme deficiency is detectable from birth. Most carriers of the defective gene remain asymptomatic throughout their lives (Lamon et al, 1979; Tschudy et al, 1980; Bottomley et al, 1981; Kappas et al, 1989). Most of the defective proteins have been shown in several studies to be inactive and variable in stability (Anderson et al, 1981; Desnick et al, 1985). Several mutations responsible for the defective PBG deaminases have been reported (Nordmann et al, 1990; Lee and Anvret, 1991; Grandchamp, 1998). Despite the genetic heterogeneity, the clinical phenotype of AIP is relatively uniform presenting with acute attacks only, and no cutaneous involvement. Usually manifests postpubertally, and degree of clinical expression is highly variable and dependant on additional precipitating factors. Excessive PBG and to a lesser extent ALA excretion in urine. Clinical syndrome more frequently manifests in females (Stein and Tschudy, 1970). The diagnosis of latent gene carriers and prognosis of the disease improved as a result of the better understanding of the genetic defects underlying the biochemical and clinical features of AIP, and it appears that the use of DNA analysis is a more reliable approach to diagnosis (Kauppinen and Mustajoki, 1992; Kauppinen et al, 1995; Puy et al, 1997).</p>			
HCP	Copro'gen Oxidase	Dominant	3q12
<p>Comment: Diminished coproporphyrinogen oxidase (CO) activity shown in leukocytes (Brodie et al, 1977b), liver (Hawk et al, 1978), lymphocytes (Elder et al, 1976; Grandchamp and Nordmann, 1977) and cultured fibroblasts (Elder et al, 1976). Dominant inheritance, homozygous form and a variant with 10% CO activity (harderoporphyria) (Nordmann et al, 1983) have been described. Skin lesions with acute attacks in about one third of patients. In absence of acute attacks the skin lesions are rare. Characterised by excessive excretion of coproporphyrin in the stool and to a lesser extent in urine (Brodie et al, 1977a; Blake et al, 1992; Schoenfeld et al, 1995). Acute attacks are more common in females (Brodie et al, 1977b; Kappas et al, 1995), and these could easily be diagnosed by the typical red fluorescence of stools under ultraviolet light. The disease is genetically heterogeneous, and different mutations identified in the CO gene of HCP patients have been reported (Martasek, 1998; Susa et al, 1998). Expression of the defective CO genes in Epstein-Barr virus transformed lymphoblasts revealed reduction in CO activity ranging from less than 5% to approximately 50% of normal (Sassa et al, 1997).</p>			
VP	Proto'gen Oxidase	Dominant	1q22-23
<p>Comment: Discussed in detail in the following section.</p>			

The Non-acute Porphyrrias

CEP	Uro`gen Cosynthase	Recessive	10q25.2-26.3
-----	--------------------	-----------	--------------

Comment:

Uroporphyrinogen-III cosynthase activity is markedly reduced, demonstrated in erythrocytes and skin fibroblasts (Romeo and Levin, 1969). Manifests from infancy. Severely photomutilating (Nordmann and Deybach, 1982). Characterised by accumulation of uroporphyrin-I and coproporphyrinogen-I in erythrocytes, plasma, bone marrow, urine and stool (Desnick et al, 1998). The type I isomers of the intermediate 7-, 6-, and 5-carboxylate porphyrins are also predominantly excreted. Anaemia and cutaneous involvement are the major debilitating features of this disease. Several mutations responsible for the defective uroporphyrinogen-III cosynthases have been identified in the genes of patients with CEP, and these have been reviewed by Desnick et al (1998).

PCT	Uro`gen Decarboxylase	Dominant	1p34
-----	-----------------------	----------	------

Comment:

Is the most common porphyria worldwide which presents with cutaneous photosensitivity and is caused by the deficiency of uroporphyrinogen decarboxylase activity (Kushner et al, 1976; Elder, 1998). Is broadly subdivided into two main types that is, sporadic (type I) and familial (type II) based on measurement of erythrocyte enzyme activity (De Verneuil et al, 1978). Main biochemical features are elevated urinary and plasma uroporphyrinogen and heptacarboxylic porphyrin. Hexa-, pentacarboxylic and coproporphyrin are elevated to a lesser extent. There is elevation of stool porphyrins in addition to a striking elevation of isocoproporphyrin. The clinical and biochemical changes can be reversed by either iron depletion or low-dose chloroquine treatment. The disease can be classified into different forms based on the underlying cause for the reduced enzyme activity and the clinical presentation:

I. Familial Form:

Decreased uroporphyrinogen decarboxylase activity which is inherited in an autosomal dominant fashion was demonstrated in liver cells (Elder et al, 1978) and erythrocytes (de Verneuil et al, 1978; McManus et al, 1988; Elder et al, 1989; Held et al, 1989; Koszo et al, 1992). Low clinical penetrance, with less than 10% subjects developing symptoms even though uroporphyrinogen decarboxylase is half normal in all tissues (de Verneuil et al, 1978; Elder et al, 1989; Elder, 1998). Mutations responsible for the defective uroporphyrinogen decarboxylase proteins have been identified in the gene sequence (Garey et al, 1989; McManus et al, 1996; Sorkin et al, 1996; Moran-Gimenez et al, 1996). Both homozygous and compound heterozygous forms exist (McManus et al, 1996; Moran-Gimenez et al, 1996).

II. Hepatoerythropoietic porphyria (HEP)

There is reduction of uroporphyrinogen decarboxylase activity by at least 75%, and severe photosensitivity from childhood which results from sustained overproduction of porphyrins (Roberts et al, 1995a; Moran-Gimenez et al, 1996). Different mutations have been identified in the uroporphyrinogen decarboxylase gene (Elder and Roberts, 1994; Meguro et al, 1994; McManus et al, 1996; Moran-Jimenez et al, 1996) and only one of these was reported to be associated with the overt clinical disease (Roberts et al, 1995a). Patients are either homozygotes or compound heterozygotes for the uroporphyrinogen decarboxylase mutations. It has been proposed that the other mutations may be autosomal recessive (Meguro et al, 1994; Roberts et al, 1995). Presenting feature is elevated erythrocyte Zn-protoporphyrin (Smith, 1986).

III. Acquired Forms:**- Sporadic**

Appears as an unusual response to alcohol abuse, sex steroids and some forms of iron overload including "Bantu siderosis" (Pimstone, 1982). Reduced uroporphyrinogen decarboxylase activity appears to be restricted to the liver (Elder et al, 1985).

- Toxic

Appears in response to certain toxic chemicals, particularly polyhalogenated hydrocarbons (Silbergeld and Fowler, 1987), includes "Turkish" porphyria (Schmid, 1960). Development of the disease appears to be a predictable and dose-related response to the absorption of a porphyrinogenic chemical (Elder, 1998).

- Chronic renal failure

Appears in a small number of patients with chronic renal failure on haemodialysis (Day and Eales, 1982). Must be distinguished from "pseudoporphyria" which resembles PCT clinically but without evidence of uroporphyrinogen decarboxylase deficiency (Moore et al, 1987).

EPP	Ferrochelatase	Dominant	18q21.3
-----	----------------	----------	---------

Comment:

Diminished ferrochelatase activity found in all tissue types (Bloomer et al, 1982). Dominant inheritance with partial penetrance (Went and Klasen, 1984). Highly variable clinical expression (Poh-Fitzpatrick, 1985). Cutaneous involvement. Manifests from infancy. Association of the cutaneous involvement with progressive liver failure is more serious, and focal deposits of protoporphyrin were demonstrated in hepatocytes and thrombi within biliary canaliculi (Thompson et al, 1973; Klatskin and Bloomer, 1974; Matilla and Molland, 1974; MacDonald et al, 1981; Sarkany and Cox, 1995). Outstanding biochemical feature is the elevated erythrocyte free protoporphyrin. Molecular analysis revealed several mutations associated with the deficiency of ferrochelatase activity, recently reviewed by Cox et al (1998). The fact that most of the affected individuals seem to possess unique mutations in the ferrochelatase gene displays the mutational heterogeneity of this condition (Rufenacht et al, 1998; Frank et al, 1999).

Variegate Porphyria

VP is an autosomal dominant disorder of haem biosynthesis which results in decreased activity of PPO, the penultimate enzyme of this pathway. Tissues from VP patients such as fibroblasts (Brenner and Bloomer, 1980b); leukocytes (Viljoen et al, 1983; Deybach et al, 1981; Boyle et al, 1986; Moore et al, 1987; Timonen et al, 1990; Guo et al, 1991); and Epstein-Barr virus transformed lymphoblasts (Meissner et al, 1986; 1993) express approximately 50% of the enzyme activity. VP is characterised clinically by photosensitive skin disease and the propensity to develop a life threatening acute neurovisceral crisis.

History and Prevalence of Variegate Porphyria in South Africa

The history of acute porphyrias in South Africa dates back to a publication in *Ōnyanga* (a journal of medical students) describing this condition, by two medical students, Eales and Chait (1939). Subsequently, a series of cases of this condition was described within the South African White population (Barnes, 1945; 1951; Dean and Barnes, 1958; 1959). Important characteristics of this condition were described in the meticulous genealogical investigations carried out by Dean (1971). In these studies Dean hypothesised that the South African VP subjects were descendants from the marriage between a Dutch couple which took place at the Cape of Good Hope in 1688. Ariaantjie Adriaanse, an orphan from Rotterdam (Netherlands) arrived at the Cape in 1688 and married one of the Free Burghers, Gerrit Jansz van Deventer. Four of their eight children inherited the genetic defect which they passed on to approximately 50% of their offspring (Jenkins, 1997). It seems therefore that one individual was heterozygous for (or a carrier of) the gene for VP.

Population growth theories suggest that in the 1960s approximately 10-20 000 subjects were carrying the defective PPO gene introduced by this couple to the Cape (Dean, 1971; Eales et al, 1980; Day, 1986). This gene had a high prevalence in this population due to the tendency to produce large families, which had resulted in the rapid expansion of this population during the 18th century from just over 1000 to 17000 in 85 years (Jenkins, 1997). Furthermore, because of the isolation of this population, gene flow into it or immigration was relatively limited (Jenkins, 1997). Besides the White population, cases of VP have been encountered in the population of mixed ancestry who are mainly descendent from the slave population imported by the Dutch to Cape Town in the 17th and 18th century, further modified by intermarriage with other indigenous and settler populations over the years. The exceptionally high prevalence of VP in the SA population is a typical example of random genetic drift (the operation of chance factors resulting in the high [or low] frequency of a gene in a population) which is known as founder effect (Jenkins, 1997).

The extensive investigations carried out by Dean on the epidemic of inherited porphyrias in SA subjects with the so-called 'van Rooyen's skin' (referring to photosensitive skin) revealed some of the characteristics of VP (Dean, 1953; 1957; Barnes, 1951). Dean observed that all the porphyric patients were over 18 years of age at the onset of their illnesses. These patients were also excreting reddish-brown urine

and stools which were found to have elevated levels of porphyrins. Later the clinical and biochemical studies of acute porphyrias carried out by Eales (1961; 1963) clarified and confirmed the increase in the concentration of porphyrins as well as the precursors, ALA and PBG in the urine specimens of the disease during the acute phase. It was found that an average of 50% of children developed the porphyria when one of the parents was a porphyric, suggesting a classical Mendelian inheritance of this condition (Dean, 1971; Jenkins, 1990). It was also observed that females were more prone to the acute attacks than males, indicating a hormonal influence in the expression and severity of this disorder.

The excellent work by Dean, Barnes and Eales in defining this disease condition culminated in them hosting the first International Conference on the Porphyrins in Cape Town, in 1963. This conference reached an important agreement on the classification of porphyrias (Proceedings of the International conference on the Porphyrins, 1963).

Presentation and Diagnosis of Variegate Porphyria

Clinical presentation

Patients with the PPO defect do not normally present with the disease condition of VP before puberty (Dean, 1971, Kramer, 1980, Meissner and Hift, 1991). Clinically VP patients may present with photocutaneous lesions, acute neurovisceral crisis, or both (Eales et al, 1980). A significant proportion of subjects with the underlying enzyme defect remain asymptomatic throughout life. A severe diffuse abdominal pain felt through the abdomen, and often the lower back, buttocks and thighs is the most common presenting feature. Vomiting, constipation, hypertension, tachycardia, and dehydration are the other common presenting features of this disorder. They may be accompanied by a peripheral neuropathy. Some patients may present with abnormal behaviour, confusion, and agitation, although in the majority of cases this is only during the acute attack in hyponatraemic patients on pethidine (Kauppinen and Mustajoki, 1992; Hift et al, 1997; Elder et al, 1997; Kirsch et al, 1998).

The porphyrin intermediates which accumulate in the upper layers of the dermis are assumed to cause a classical photodermatitis (Runge and Watson, 1962; Day, 1986). The common hypothesis, that porphyrins on exposure to light in the near ultraviolet range ($\pm 400\text{nm}$) can cause the formation of singlet toxic oxygen species which may in turn be responsible for peroxidative destruction of cellular membranes, remains to be shown as being the primary pathogenic mechanism operative in VP. Thus other mechanisms may also contribute to the photocutaneous sensitivity (Poh-Fitzpatrick, 1998). In addition, an interesting specific observation in VP is that uro- and heptacarboxylic porphyrin rather than the predominant excreted proto- and coproporphyrin species are present in the skin (Day, 1986).

The skin lesions are generally in the form of blisters, erosions, fragility, milia and abnormal pigmentation which eventually leads to thickening, grooving, hirsutes and premature aging. The lesions are commonly found in sun-exposed areas of the skin

such as the dorsa of the hand, forearm and feet; the face and nape of the neck (Elder et al, 1997; Kirsch et al, 1998).

Biochemical presentation and diagnosis

The classical biochemical picture of VP is characterised by an increase in faecal protoporphyrin and to a lesser extent, coproporphyrin; and the urinary porphyrin precursors, ALA and PBG which are elevated during the acute attack (Eales et al, 1980; Day, 1986; Nordmann and Deybach, 1990; Kappas et al, 1989; Hift et al, 1997). The porphyrin elevations are consistent with a partial block in the haem pathway at the level of PPO. There is evidence to suggest that the accumulation of ALA and PBG during the acute attack of VP, may be due to the allosteric inhibition of PBG deaminase by the accumulating protoporphyrinogen and coproporphyrinogen (Meissner et al, 1993).

Thus biochemical diagnoses may be based on the quantitation and analysis of excreted porphyrins, and in the case of a suspected acute attack on the measurement of the porphyrin precursors. The experience of our laboratory also suggests that the finding of 5-COOH-porphyrin or "pseudo 5-COOH-porphyrin" compounds in VP patients is of diagnostic significance (Moore et al, 1987; Hift et al, 1997).

In addition to the classical porphyrin extraction and chromatographic techniques, a plasma protein-porphyrin complex gives a characteristic peak of fluorescence emission between 621 and 627 nm at an excitation of 405 nm, and appears specific for VP diagnosis (Poh-Fitzpatrick, 1980; Long et al, 1993; Gregor et al, 1994; Da Silva et al, 1995). This method is fast replacing the more traditional porphyrin chromatographic techniques in many parts of the world.

The enzyme assay on PPO activity, which can be performed on Epstein-Barr virus transformed lymphoblasts (Meissner et al, 1986) and fibroblasts (Brenner and Bloomer, 1980a) to demonstrate the 50% decrease in enzyme activity, is not suitable for diagnostic purposes due to the low levels of this activity in these cell types and the tediousness of the assay.

While it is not perhaps relevant to review the finer intricacies of the diagnosis of VP in detail here, the following are interesting observations.

1. The profile of excreted porphyrin varies widely between and within individual VP patients. Chromatographic analysis of porphyrin profiles may indeed be normal or near normal in some VP patients. About 30-40% of VP patients examined in our laboratory, either consistently or transiently, present with normal porphyrin excretory profiles (Kirsch et al, 1998). The biochemistry of many of the older patients who presented with elevated porphyrin excretion in early adult life sometimes return to normal levels.

2. The stool porphyrin profile has been shown to be normal in some VP patients in the presence of elevated biliary porphyrin concentrations (Logan, et al, 1991). Also, dissimilarities in stool and biliary porphyrin excretion profiles have been demonstrated in normal subjects in which coproporphyrin and protoporphyrin

predominate in bile and stool, respectively (Hift R., unpublished data). This unexplained paradox may be due to the differential reabsorption of porphyrins in the small intestine or it may be due to bacterial metabolism within the colon.

Genetic presentation and diagnosis

The isolation of the cDNA (Nishimura et al, 1995b; Dailey and Dailey, 1996b) and characterisation of the gene (Roberts et al, 1995b) encoding human PPO has facilitated the identification of many mutations. Indeed, 81 mutations responsible for the defective PPO genes in VP have been reported worldwide to-date (Deybach et al, 1996; Meissner et al, 1996; Warnich et al, 1996a; Frank et al, 1998a, b, c, d, e; Roberts et al, 1996, 1998; Lam et al, 1996, 1997; Puy et al, 1996; Corrigall et al, 1998, 2000, de Rooij et al, 1997, Kauppinen et al, 1996, Whatley et al, 1999). This has led to the development of DNA diagnostic techniques, which have been applied in the management of families with this condition. Because VP has a variable clinical and biochemical expression, and the fact that the disease seldom manifests before puberty, its diagnosis is significantly enhanced by the use of mutational analysis. Indeed, in SA screening for the R59W mutation is useful as this defect appears to be accountable for over 90% of VP in this country (Meissner et al, 1996; Warnich et al, 1996a). However, a major limiting factor to the role of mutational analysis in the diagnosis of VP is the genetic heterogeneity of this condition in the rest of the world. Even in South Africa, in spite of the founder gene effect, nine different mutations have been identified to-date (Meissner et al, 1996, Warnich et al, 1996a, Hift et al, 1997, Corrigall et al, 1998, 2000).

A small number of cases of apparently homozygous VP (HVP) have been reported worldwide including South Africa (Korda et al, 1984, Murphy et al, 1986, Mustajoki et al, 1987, Norris et al, 1990, Gandolfo et al, 1991, Coakley et al, 1990, Hift et al, 1993, Roberts et al, 1998, Corrigall et al, 2000). These individuals have a marked reduction of PPO activity [$<25\%$ of normal] (Hift et al, 1993, Roberts et al, 1998), and a severe phenotype with onset occurring mainly in infancy. However a case of late presentation (at age 19 years) has been documented (Corrigall et al, 2000). Typical symptoms include growth retardation, brachydactyly, nystagmus, mental retardation and convulsions. Prior to the identification of the PPO gene many of these cases were thought to represent VP homozygosity, thus the syndrome became known as HVP. Nine of the thirteen cases of apparently homozygous VP, reported to date, have been investigated at a molecular level. Only two of these cases were homozygotes (Roberts et al, 1998) while the remainder were compound heterozygotes (Roberts et al, 1998, Meissner et al, 1996, Frank et al, 1998c, Kauppinen et al, 1996, Corrigall et al, 2000).

Interestingly, in South Africa the gene frequency of the R59W mutation is such that homozygosity would have been expected to occur (Jenkins, 1996). No cases of R59W homozygosity have been reported to date. Thus, it would appear that homozygosity for this common South African mutation is lethal (Corrigall et al, 2000).

On examination of the above it stands to reason that:

1. Intensive screening of VP families which leads to identification of carriers, and the better education of both patients and doctors on factors precipitating acute attacks, should result in a significant reduction of the number of VP subjects who present with the life threatening acute attacks.
2. When possible VP individuals should be described in terms of their genotype, biochemical activity, and clinical expression in order to allow easier comparison and critical understanding of data emanating from all parts of the world.

General Comments

On reading the above it is clear that the application of molecular biological techniques in the study of the haem biosynthetic proteins has yielded valuable information and facilitated their characterisation. The genes or cDNA sequences have now been isolated and characterised for all of the human enzymes involved in the haem pathway. These have led to refinement of purification procedures enabling simple rapid purifications to be carried out with large protein yields, which in turn have allowed detailed characterisations, and in some cases, crystal structures to be determined. The new methodologies have also helped clarify some of the ambiguities encountered when these proteins were purified by conventional chromatographic purification procedures. These advances have also facilitated the correct chromosomal mapping of some of these genes in the human genome, improving dramatically on previous methods used for this purpose.

Importantly, these methods have become useful and additional tools in investigating the factors associated with the pathogenesis of porphyrias. Many mutations which are responsible for the defective enzymes in the inherited porphyrias have now been identified and characterised.

Despite the advances mentioned above difficulties may still be encountered. Indeed, PPO is still difficult to purify to homogeneity despite its cloning and the use of affinity chromatography purification procedures. This has contributed to the lack of information on structure-function relationship of this enzyme. Continued interest in PPO is due to its role in the pathogenesis of VP and the fact that PPO is the molecular target for several peroxidizing herbicides. This has raised much interest and stimulated a lot of research by the agricultural sector aimed at exploiting this property in crop control (Matringe et al, 1989a, b; Witkowski and Halling, 1988). The next chapter reviews PPO in detail.

Chapter Two

Literature Review Continued

As this thesis is largely concerned with work on the protein PPO it is reviewed in relevant detail in this chapter.

Protoporphyrinogen Oxidase (PPO) and the Conversion of Protoporphyrinogen-IX to Protoporphyrin-IX.

PPO is the last enzyme common to both the haem and chlorophyll pathways (Jacobs and Jacobs, 1981). At this, the penultimate step, the pathway leading to the formation of these two compounds diverges, with specific chelatases inserting either iron (Fe^{2+}) or magnesium (Mg^{2+}) into the protoporphyrin tetrapyrrole to form haem and chlorophyll precursors, respectively (Beale and Weinstein, 1990).

Briefly, during the six electron oxidation catalysed by PPO, the methylene bridges ($-\text{CH}-$) in protoporphyrinogen-IX are converted into methenyl bridges ($-\text{CH}=\text{}$) and protoporphyrin-IX results. This is depicted in figure 2.1.

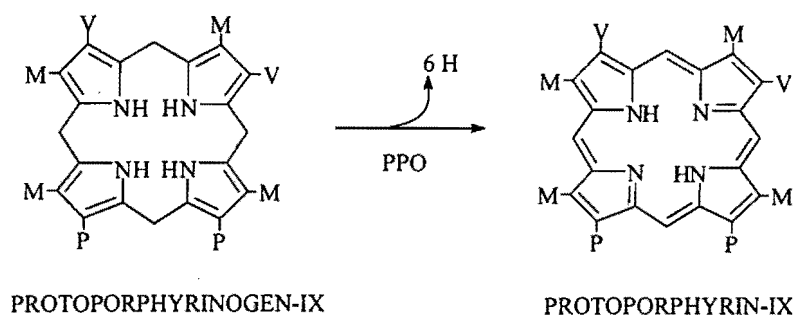


Figure 2.1 The penultimate step of haem biosynthesis involves six electron oxidation of protoporphyrinogen-IX to protoporphyrin-IX by protoporphyrinogen oxidase. In the presence of air protoporphyrinogen-IX is auto-oxidised to protoporphyrin-IX. M = methyl, V = vinyl and P = propionate.

It must be added that in the presence of oxygen protoporphyrinogen-IX is auto-oxidised into the highly conjugated protoporphyrin-IX molecule in a light-dependent non-enzymatic (chemical) reaction, thus compounding the difficulties associated with work on this enzyme. This *in vitro* oxidation of protoporphyrinogen-IX occurs rapidly at neutral and acidic pH. However, under normal circumstances in cells this reaction requires enzymatic catalysis due to the reducing environment in the mitochondria (Porra and Falk, 1964; Meissner, 1990).

Although all PPOs share a common feature in that they oxidise protoporphyrinogen-IX to protoporphyrin-IX, these proteins show inter- and sometimes intra-species diversity in their kinetic and other physicochemical properties. It must also be borne in mind that some of the apparent diversities could have arisen because of the differences between earlier and later more definitive reports. These can conceivably arise as early workers did not have the advantage of known and identifiable protein or cDNA sequence data. Thus some apparently discrepant reports could have been artefactual (eg. Some of the "PPO" apparently purified by Siepker et al (1987) was probably α -1-antitrypsin – personal communication L. Siepker, S. Kramer).

Early work on the yeast (*Saccharomyces cerevisiae*) reaction (Poulson and Polglase, 1975) and subsequent work on the mammalian mitochondrial reaction (Poulson, 1976) provided much of the information which supported the specific enzymatic catalysis of this step in haem and chlorophyll biosynthesis. The isolation and subsequent characterisation of protoporphyrinogen-IX oxidising proteins from several sources such as yeast (Poulson and Polglase, 1975), rat livers (Poulson, 1976), the photosynthetic bacterium, *Rhodospseudomonas spheroides* (Jacobs and Jacobs, 1981), and barley (Jacobs and Jacobs, 1984a) provided conclusive evidence for the enzymatic catalysis of the penultimate step in haem biosynthesis. Jacobs and Jacobs, (1984b) demonstrated the existence of this protein in several prokaryotic and eukaryotic species such as rat, barley, spinach, *Escherichia coli* and *R. spheroides*.

Anaerobic enzymatic oxidation of protoporphyrinogen-IX was demonstrated by the isolation of this protein from the obligate anaerobic bacterium, *Desulfovibrio gigas* (Klemm and Barton, 1985; 1987). PPO activity was solubilised from the plasma membranes of *D. gigas* and the protein was purified to apparent homogeneity (Klemm and Barton, 1987).

Terminal Electron Acceptors in the Oxidation of Protoporphyrinogen-IX to Protoporphyrin-IX

Consideration of the reaction catalysed by PPO, implies that there is an electron acceptor required for catalysis to occur. It appears that during aerobic oxidation of protoporphyrinogen-IX to protoporphyrin-IX, by both eukaryotic and prokaryotic PPOs, molecular oxygen is utilised as the terminal electron acceptor (Poulson, 1976; Deybach et al, 1985). Aerobic bacteria such as *Bacillus subtilis* (Dailey et al, 1994a; Hansson and Hederstedt, 1994b) and *Myxococcus xanthus* (Dailey and Dailey, 1996a), also utilise molecular oxygen as the final acceptor of the electrons. In some facultative prokaryotes such as *E. coli*, oxygen serves as the final acceptor of the electrons released from the aerobic reaction, but in the anaerobic oxidation alternative electron acceptors such as nitrate and fumarate are utilised (Jacobs and Jacobs, 1975; 1976). Facultative organisms are defined as those organisms that can exist and survive well under either aerobic (in presence of oxygen) or anaerobic (in absence of oxygen) conditions. In these organisms it is proposed that the electrons removed from the oxidation of protoporphyrinogen-IX are transferred to the electron transport chain (Jacobs and Jacobs, 1977a); thus providing a source of energy regeneration. The coupling of the oxidation reaction to the nitrate/fumarate reductase system was shown to involve the use of quinones as electron carriers (Jacobs and Jacobs, 1977 a & b).

However, different facultative organisms utilise different alternative terminal electron acceptors in the anaerobic reaction. Nitrate and fumarate were ineffective as electron acceptors in the anaerobic reaction of *Staphylococcus epidermidis*, which is a facultative anaerobe (Jacobs and Jacobs, 1976). It seems as if in this organism protoporphyrinogen-IX oxidation is strictly an oxygen-dependent process. The anaerobic oxidation of protoporphyrinogen-IX into protoporphyrin-IX in the obligate anaerobe, *D. gigas*, was also shown to be coupled to the reduction of several physiologic electron acceptors such as; NAD^+ , NADP^+ , FAD, FMN, nitrite, fumarate and ATP plus sulphate (Klemm and Barton, 1985, 1989), and a non-physiologic compound, dichlorophenolindophenol (DCIP) (Klemm and Barton, 1987). In the photosynthetic bacterium, *R. sphaeroides*, the anaerobic reaction is similar to *E. coli* in that protoporphyrinogen-IX oxidation has been shown to be coupled to the electron transport chain utilising quinones as electron carriers (Jacobs and Jacobs, 1981).

Due to the different conditions under which reactions take place in prokaryotes, on many occasions different isoforms of the same enzyme are often used in the catalysis of the same reaction. But such isoforms which differ on the basis of their oxygen requirements have not been unequivocally identified in PPOs. While, it has been suggested that the two PPOs identified in *E. coli* that is, hemG (Sasarman et al, 1993, Nishimura et al, 1995a) and hemK (Nakayashiki et al, 1995) could be PPO isoforms which operate under aerobic and non-aerobic conditions of protoporphyrinogen oxidation this appears not to be the case.

pH Conditions for the Enzymatic Oxidation of Protoporphyrinogen-IX

Generally PPOs operate optimally at and above neutral pH. *R. sphaeroides* (Klemm and Barton, 1987), *E. coli* (Sasarman et al, 1993), *S. cerevisiae* (Poulson and Polglase, 1975), spinach, barley (Jacobs and Jacobs, 1984a), potato and maize (Camadro et al, 1991) have pH optima in the range 7.0 to 7.5. Interestingly, the oxygen-independent *D. gigas* enzyme oxidised protoporphyrinogen-IX optimally over a wide pH range from 7.0 to 11 (Klemm and Barton, 1987). The fact that this PPO is a very large multisubunit protein complex may be contributing somehow to the pH tolerance over such a wide range. Thus, the interaction between the six subunits which comprise the active enzyme could provide some protective buffering effect which minimises the effects of pH change over this range on the enzyme's catalytic activity.

Mammalian PPOs such as the rat (Poulson, 1975), bovine (Siepker et al, 1987), human (Camadro et al, 1985) and mouse (Dailey and Karr, 1987) have pH optima of 8.1-8.8; 8.7; 7.2 and 7.1, respectively. The purified mitochondrial and etioplasic barley PPOs had the lowest reported pH optimum range of 5-6, which indicates preference for an acidic environment (Jacobs and Jacobs, 1987). Previously, optimum PPO activity for the crude sonicated barley enzyme preparation was achieved at pH 7.3 (Jacobs and Jacobs, 1984a). Considering the fact that undesirable auto-oxidation of protoporphyrinogen-IX would be fairly rapid at such a low pH range (<6), it would be logical therefore to speculate that under physiological conditions pH 7.3 could be the operational range for this enzyme.

Isolation and Purification of PPOs

For many years researchers were dogged by difficulties in attempts at purification of this membrane bound protein using a variety of standard chromatographic methods. PPOs are hydrophobic membrane bound proteins requiring detergents for solubilisation and the various PPO forms behave differently in the presence of different detergents. Furthermore, these proteins show variable stability. In the presence of 0.2% octyl glucopyranoside purified human PPO may be stored for days at 4°C and even retains significant activity after overnight storage at room temperature (Dailey and Dailey, 1997a). In contrast, *M. xanthus* PPO appears less stable and tends to precipitate out when stored at 4°C (personal observation). Prolonged storage (1 month) of yeast mitochondrial membranes at -80°C resulted in degradation of PPO into immunologically detectable low molecular weight fragments. Proteolysis was however significantly less than when stored at -20°C (Camadro et al, 1994). Finally the assay for PPO activity is extremely laborious requiring fresh preparation of the substrate which involves sodium amalgam reduction of protoporphyrin-IX and, since porphyrins are photo-degradable compounds, the PPO assay is performed in the dark. Despite these difficulties a number of laboratories managed to build up a considerable knowledge of this enzyme, even though the data was sometimes obtained using crude sonicated enzyme preparations, or partially purified preparations.

Efforts by various researchers working on this protein paid off with achievements of partial and on very rare occasions homogeneous purification, and subsequent characterisation of PPOs from various sources such as rat (Poulson, 1976); *S. cerevisiae* (Poulson and Polglase, 1975; Camadro et al, 1994); *R. sphaeroides* (Jacobs and Jacobs, 1981) barley (Jacobs and Jacobs, 1987; Jacobs et al, 1989); *D. gigas* (Klemm and Barton, 1987); mouse (Dailey and Karr, 1987; Ferreira and Dailey, 1988; Proulx and Dailey, 1992); bovine (Siepker et al, 1987); spinach (Matringe et al, 1992a); and potato plant (Johnston et al, 1998). In many of these efforts a combination of several different steps of standard chromatographic purification techniques were applied to improve purification of the protein. This resulted in long procedures and the protein yield was very low making it difficult to carry out lengthy characterisation studies. The rapid degradation of this protein under cold storage compounded this problem.

More recently several PPO genes have been cloned and sequenced from various species. These are discussed later in this chapter. However, pertinent to this discussion is the fact that cloning of the various PPO genes and overexpression of this protein in *E. coli* cells has resulted in the establishment of simple rapid purification procedures enabling researchers to produce large amounts of the protein. This breakthrough facilitated confirmatory and further characterisation of this enzyme from various sources. In spite of this data, information on the protein structure, and in particular the understanding of the catalytic mechanism at the enzyme active site is still lacking.

PPO Protein Size Range

Most oxygen-dependent PPOs, such as the human (Nishimura et al, 1995; Dailey and Dailey, 1996b); mouse (Taketani et al, 1995a, Dailey et al, 1995); *M. xanthus* (Dailey and Dailey, 1996a); *B. subtilis* (Hansson and Hederstedt, 1992; Dailey et al, 1994a); *S. cerevisiae* (Camadro et al, 1994); *A. thaliana* (Narita et al, 1996); bovine (Siepker et al, 1987); tobacco plant (Lermontova et al, 1997); spinach (Matringe et al, 1992); and potato plant (Johnston et al, 1998) forms have reported molecular weights within the range 51 000-57 000 Da. The rat enzyme is the smallest reported mammalian PPO with a molecular weight of 33 000 Da determined by SDS-PAGE (Poulson, 1976). This is similar to the molecular weight of 36 000 Da reported for the purified barley PPO (Jacobs and Jacobs, 1987). However, as both these are early reports they may well be spurious. The 21 200 Da *E. coli* PPO (Sasarman et al, 1993) and 148 000 Da reported for the *D. gigas* multisubunit enzyme (Klemm and Barton, 1987) represent anaerobic enzymatic oxidation and are distinct from the oxygen-utilising forms of the enzyme.

Subunit Composition of PPO Proteins

Analysis of various purified PPOs suggest that most of these proteins exist as either monomers such as the *B. subtilis* (Dailey et al, 1994a); *S. cerevisiae* (Camadro et al, 1994); and mouse (Dailey and Karr, 1987); or homodimers such as *M. xanthus* (Dailey and Dailey, 1996a) and human PPOs (Nishimura et al, 1995b; Dailey and Dailey, 1996b). The oxygen-independent *D. gigas* enzyme is a large multi-subunit protein complex of 148 000 Da. The active form of this protein is a hexamer composed of two of each of three different subunits of 12 000 Da, 18 500 Da, and 57 000 Da which are held together by disulfide bonds (Klemm and Barton, 1987).

PPO Cofactor Composition

Evidence from spectral analysis and gene/protein sequence information shows that these enzymes are flavoproteins. Such evidence has come in the form of flavins extracted from various purified PPOs and the identification of the dinucleotide binding ($\beta\alpha\beta$) motif near the N-terminal sequences of cloned PPO genes. Generally the flavin cofactors are non-covalently bound to these proteins. The bovine FAD (Siepker et al, 1987) cofactor is an exception in that it appears to be covalently associated with the protein.

When Poulson and Polglase (1975) first partially purified and characterised the yeast PPO they observed an absorption peak at 410 nm which seemed to indicate presence of a prosthetic cofactor. However, no flavins were detected in their enzyme preparations. Interestingly, studies of the oxidation of protoporphyrinogen-IX by the obligate anaerobic bacterium, *D. gigas*, showed that in the presence of several electron acceptors such as NAD^+ , NADP^+ , FAD, and FMN this reaction was significantly stimulated (Klemm and Barton, 1985). This suggested a role of these compounds in the enzymatic activity, but unfortunately this property was not investigated further in this organism.

Spectral analysis of a purified bovine PPO revealed a typical flavoprotein spectrum which was indicative of the association of this enzyme with a flavin cofactor (Siepker et al, 1987). Subsequent characterisation of the extracted flavin cofactor indicated that it was FAD. The extraction of the cofactor required the use of hot mineral acids and trypsin digestion demonstrating that it was covalently bound to the protein. The use of exogenous flavins to try and stimulate the enzyme activity proved unsuccessful, and Siepker et al (1987) attributed this to the requirement for tight covalent binding of the cofactor by the enzyme. An unusual finding with the bovine PPO FAD cofactor was that it appeared to be present in a reduced form in the active enzyme (Siepker et al, 1987), a situation which would be expected to occur only when the electrons are released from the porphyrinogen during the catalysis of the reaction by the enzyme.

On the other hand, Dailey and Karr (1987) did not find any evidence of a chromophoric cofactor from the ultraviolet/visible spectral analysis of the purified mouse PPO. A fluorescence emission spectroscopy at an excitation wavelength of 450 nm under both oxidizing and reducing conditions did not yield a typical flavin spectrum, suggesting that this purified PPO was not a flavoprotein. When the effects of several electron acceptors such as FAD were tested on this enzyme no significant stimulation could be detected (Dailey and Karr, 1987; Ferreira and Dailey, 1988). In a subsequent re-investigation of this property on this enzyme by the same laboratory a flavin moiety was identified in the purified protein from ultraviolet/visible spectral analysis (Proulx and Dailey, 1992). This group used a shorter and more rapid scheme for the purification of the mouse PPO which gave higher protein yields than in previous procedures. Lack of any effect on oxidation of the purified enzyme preparation indicated that the cofactor was present in an oxidised state. The fluorescence emission spectrum of the isolated cofactor gave the same emission maximum as an FMN standard, suggesting that this was the cofactor associated with this enzyme. This was also supported by data from HPLC analysis. Attempts to reconstitute the activity of this enzyme with exogenous flavins were not successful. Later, the presence of the dinucleotide binding motif and identification of FAD as a cofactor in mammalian PPO were reported for the first time in the mouse (Dailey et al, 1995) and human (Dailey and Dailey, 1996b) enzymes.

During the cloning and characterisation of the *B. subtilis hemY* cDNA sequence Dailey et al (1994a) identified the dinucleotide binding domain in this PPO gene, suggesting association of this enzyme with flavins. Subsequent spectral analysis of the purified *B. subtilis* PPO revealed a typical flavoprotein spectrum (Hansson et al, 1997).

At that time an improved purification of the yeast PPO by Camadro et al (1994) also revealed the presence of a flavin cofactor which was identified by its pH-dependent spectral shift to be FAD. The cofactor was present at a flavin-to-protein ratio of 1. Slightly later, sequence analysis of the cloned yeast *hem14* gene coding for PPO, revealed a putative $\beta\alpha\beta$ motif (Camadro and Labbe, 1996). A typical flavin absorption spectrum was obtained from spectrophotometric analysis of the purified protein and extracted cofactor.

To further confirm the flavoprotein nature of the *B. subtilis* PPO Hansson et al (1997)

FAD-containing enzymes. In the presence of FAD the enzymatic activity was also stimulated 2-3 fold. This data suggested a role of the FAD cofactor in enzyme activity. The cloned PPO from another aerobic bacterium, *M. xanthus*, was also reported to be a flavoprotein that was non-covalently associated with FAD (Dailey and Dailey, 1996a). Analysis of the PPO cDNA sequence from this organism also revealed the presence of the dinucleotide binding motif. In this protein the cofactor was reported to be present at a ratio of one FAD per homodimer or 0.5 FAD per protein subunit.

Nishimura et al (1995a) had also identified the flavodoxin motif in the *hemG* cDNA sequence of *E. coli* which suggested that this enzyme was a flavoprotein.

Consideration of the cDNA sequence data from many species reveal that human (Nishimura et al, 1995b), mouse (Taketani et al, 1995a), plant (Narita et al, 1996), yeast (Camadro and Labbe, 1996) and some bacterial (Dailey and Dailey, 1996a, 1998) PPO sequences share a high degree of homology near the amino terminus. This area corresponds to a typical dinucleotide binding ($\beta\alpha\beta$) motif. This is a defined, conserved sequence which occurs in the form of a GXGXXG fingerprint, comprised of three glycine (G) residues and other amino acid (X) substituents which include an acidic residue, found near the N-termini of many NAD and flavin binding protein domains (Wierenga et al, 1986). Both DNA sequence analysis for the dinucleotide binding motif and further extraction of these cofactors from purified proteins have facilitated the identification of PPOs as flavoproteins associated with either FAD or FMN as cofactors.

Plant biologists followed a similar path of discovery. Fluorometric analysis of a crude plant PPO (barley) preparation looking for cofactors involved in the electron transfer process showed no association of this protein with any chromophoric compounds (Jacobs and Jacobs, 1987). On subsequent analysis of the purified barley PPO, using extraction procedures for either covalently or non-covalently bound cofactors, there was no evidence of a flavin cofactor association with this protein (Jacobs et al, 1989), suggesting that the plant PPO was not a flavoprotein. This property appears either unique to the barley PPO or represents a conclusion based on a non-PPO protein, because recent cloning of the *A. thaliana* PPO and subsequent analysis of the cDNA sequence revealed the presence of the typical dinucleotide binding motif (Narita et al, 1996). More recently the presence of this dinucleotide binding domain has also been identified in the cloned and characterised plastidal and mitochondrial PPO isoforms of the tobacco plant (Lermontova et al, 1997) and potato (Johnston et al 1998). Although the physical analysis for the flavin association with these proteins has not been done, available sequence information indicates that plant PPOs are also flavoproteins.

The identification of the flavin cofactors in association with PPOs has been an important discovery since this sheds light on the ability of the protein to transfer six electrons during the oxidation of protoporphyrinogen-IX. Sequence analysis revealed that, in addition to the dinucleotide binding motif, there is high similarity in an approximately 60 residue-long region that extends beyond this motif between PPOs, bacterial and plant phytoene desaturases (PHDs) and animal monoamine oxidases (MAOs) (Dailey and Dailey, 1998). These authors propose that this is a signature

motif for an FAD superfamily containing PPOs, PHDs and animal MOAs as this region of similarity does not appear in any of the other proteins in current gene sequence databases.

Substrate Specificity

Naturally, PPOs catalyse the oxidation of protoporphyrinogen-IX into protoporphyrin-IX. Some PPOs such as the barley and spinach (Jacobs and Jacobs, 1984a), *B. subtilis* (Dailey et al, 1994a), rat (Poulson, 1975), mouse (Dailey and Karr, 1987), and human enzymes (Camadro et al, 1985) also oxidise the non-physiological dicarboxylic mesoporphyrinogen-IX. Mesoporphyrinogen-IX is normally oxidised at a slower rate than protoporphyrinogen-IX, but some of the enzymes oxidise the two compounds at a similar rate, e.g. spinach and barley enzymes (Jacobs and Jacobs, 1984a). Both these compounds are dicarboxylic tetrapyrroles which are structurally similar and this explains their recognition as substrates by the same enzyme.

The *B. subtilis* enzyme is exceptional to all the known PPO species in that, in addition to protoporphyrinogen-IX and mesoporphyrinogen-IX, it was shown to oxidise the four carboxylic coproporphyrinogen-III molecule (Hansson and Hederstedt, 1994; Dailey et al, 1994a), which is the substrate in the previous reaction of the haem synthetic pathway catalysed by coproporphyrinogen oxidase. The fact that *B. subtilis* PPO oxidises coproporphyrinogen-III into coproporphyrin-III (Hansson et al, 1997) indicates that a true coproporphyrinogen oxidase, which catalyses the oxidative decarboxylation of coproporphyrinogen-III into protoporphyrinogen-IX, exists in this organism. Indeed, the recent cloning of the *hemN* gene from *B. subtilis* which encodes an oxygen-independent coproporphyrinogen-III decarboxylating protein (Hippler et al, 1997) supports this.

Besides protoporphyrinogen-IX, the PPO of the photosynthetic bacterium, *R. sphaeroides* was reported to utilise both mesoporphyrinogen-IX and uroporphyrinogen-I as substrates (Jacobs and Jacobs, 1981). However, broad substrate specificity is not a general prokaryotic PPO property as demonstrated by *M. xanthus* protein (Dailey and Dailey, 1996a) which has high specificity for protoporphyrinogen-IX. Despite its uniqueness the *B. subtilis* PPO is not a general porphyrinogen oxidase, because it did not oxidise the eight-carboxylic, uroporphyrinogen-III (Hansson and Hederstedt, 1994a).

Sub-cellular Localisation and Translocation of PPO

Several approaches were used in the localisation of PPO within the cells of various organisms. Investigation methods used can broadly be grouped into three, viz.

1. Assaying PPO activity in subcellular or suborganellar fractions separated by differential centrifugation methods (Jacobs et al, 1982; Deybach et al, 1985).
2. Identification of PPO in isolated subcellular or organellar fractions using specific labelled PPO ligands (Birchfield and Casida, 1996).

3. *in vitro* translation and import of cloned PPO, expressed in *E. coli* cells, into isolated mitochondria and chloroplasts (Nishimura et al, 1995b, Lermontova et al, 1997).

The work of Poulson and Polglase (1975) and Poulson (1976) demonstrated that both the yeast and mammalian PPO activities were associated with mitochondrial membranes and required detergents for solubilisation. In plants the enzyme activity was demonstrated in mitochondria as well as chloroplasts (Jacobs et al, 1982). The extensive investigation carried out by Deybach et al (1985) on rat livers presented evidence which showed that PPO was an intrinsic protein of the inner mitochondrial membrane. This involved subfractionation of purified mitochondria using the digitonin method, tryptic digestion with and without detergent solubilisation, and chemical modification with diazobenzene sulfonate of intact and sonicated mitoplasts. Ferreira et al (1988) used a membrane-impermeable water soluble inhibitor of PPO, the ditaurine conjugate of bilirubin, and reported inhibition of rat liver PPO activity in intact mitochondria and mitoplasts, and also in sonicated mitochondria. These findings demonstrated that the PPO catalytic site was on the cytosolic side of the inner membrane since this membrane was impermeable to this inhibitor.

Smith et al (1993) performed differential subfractionation of cells from pea leaves, into mitochondria and chloroplast fractions and showed that PPO activity was present in these organelles at a ratio of 1:2, respectively. Previously, Matringe et al (1992a) had shown that in subfractionated chloroplasts PPO activity was found in thylakoids and the total envelope membranes, but not in the stroma. The relative PPO activity per mg total protein was 7-8 times higher in the envelope membranes compared to thylakoids. Using subfractionated yeast mitochondria, Camadro et al (1994) also demonstrated that PPO activity was localised at the inner membrane of this organelle.

The progress made in the identification and cloning of PPO genes allowed new methods to be applied in further confirming the localisation of these proteins within cells. These involved the *in vitro* transcription and translation of cloned PPO genes, and the import of the labelled translation products into isolated mitochondria and chloroplasts. The mitochondrial importation of these products was confirmed by the reisolation of the product from these organelles and comparing its properties with those of the known purified protein. Nishimura et al (1995b) performed an *in vitro* transcription of the human PPO gene and translated the mRNA in the rabbit reticulocyte lysate system in the presence of [³⁵S]methionine to produce a labelled protein product. The labelled translation product was imported into isolated intact mouse mitochondria. Reisolation of the labelled protein yielded a protein with similar molecular weight to that predicted for the human PPO gene, suggesting that this protein did not undergo modification on insertion into the mitochondrial membrane. Within the mitochondrial membrane this protein was protected against proteolytic digestion in the absence of detergents. On addition of 0.3% Triton X-100 digestion of the protein occurred demonstrating the intrinsic association of this protein with the membrane lipid bilayer.

In a similar study, Lermontova et al (1997) demonstrated the importation of the tobacco plant plastidal and mitochondrial PPO isoforms into intact chloroplasts and mitochondria (isolated from pea plant), respectively. These investigators found that

the plastidal PPO isoform was processed from a 59 000 Da precursor protein into a 53 000 Da mature form which was protected against protease degradation. No processing was observed in the mitochondrial isoform which was also protected against protease degradation, indicating that these proteins were hidden within the membrane lipid bilayer. The correct targeting of these PPO isoforms was confirmed by immunodetection in plastids and mitochondria using specific antibodies.

Computer analysis of several PPO gene or protein sequences has revealed the lack of membrane targeting leader sequences or obvious internal targeting signals in these proteins (Dailey et al, 1994a, Nishimura et al, 1995, Dailey and Dailey, 1996a, b). The amino terminal portion (28 amino acids) of human PPO contains features which are characteristic of a presequence in that, it has 3 basic residues and no acidic residues (Nishimura et al, 1995b). However, although this portion can form an alpha helix, it is not amphiphilic as the hydrophobic residues are not clustered on the opposite side of positively charged amino acids and the hydroxylated amino acids are scattered. Although these features may represent a mitochondrial targeting signal, human PPO may have another type of signal at its amino-terminus which has not yet been identified. The only PPO species that have been demonstrated thus far to be synthesized as precursor proteins with amino-terminal putative membrane targeting leader sequences are *Arabidopsis thaliana* (Narita et al, 1996), and the plastidal isoform of the tobacco plant enzyme (Lermontova et al, 1997). Since it has been established that PPOs are membrane bound proteins associated with the inner mitochondrial membrane, it appears therefore that those proteins which lack the leader sequences apparently possess internal membrane targeting signals which have not yet been identified.

Indeed, such unique internal targeting mechanisms have been proposed for other proteins destined for mitochondrial membranes (Lill et al, 1996, Stuart and Neupert, 1996). For example, the BCS1 protein (involved in the assembly of complex III, that is, ubiquinol-cytochrome c reductase [bc1] of the mitochondrial respiratory chain), which spans the inner mitochondrial membrane, contains a stretch of positively charged amino acids (amphiphilic segment) after a transmembrane domain sequence, which were shown to function as the internal targeting signal through the formation of an amphipathic helix (Folsch et al, 1996). It was suggested that such an amphipathic helix could react on its apolar side with the transmembrane domain and form a hairpin loop which will penetrate the translocating channel of the inner membrane to facilitate translocation. In addition to the membrane targeting sequences the inner mitochondrial membrane proteins are reported to possess so-called topogenic signals which determine not only their correct import into the membranes but also their final orientation. For instance, the cytochrome oxidase subunit Va, which spans the inner membrane, is reported to have such a topogenic signal which prevents its translocation across the inner membrane, by interacting with translocation components in the membrane, resulting in the arrest of its complete translocation (Stuart and Neupert, 1996). Since many PPOs lack any cleavable mitochondrial targeting signals their import into the membranes could be similar to that described for the BCS1 protein. Such topogenic signals are therefore likely to exist in PPO proteins though they have not yet been demonstrated.

Initially it was suggested that yeast PPO was synthesised as a high molecular weight precursor which was converted to a mature mitochondrial membrane-bound form (Camadro and Labbe, 1994). However, a later publication from this group (Camadro and Labbe, 1996) suggested that the hydropathy profile of yeast PPO demonstrated a moderately high hydrophobic protein with a single potential membrane-spanning segment (residues 13-33). Analysis revealed that this was not a transmembrane domain. There are several hydrophobic regions in yeast PPO but all are less than 15 uncharged residues and are therefore unlikely to form membrane-spanning segments. While it is possible that shorter helical domains could be responsible for the insertion of PPO into the inner mitochondrial membrane another possibility is that PPO is anchored to the membrane by a mechanism such as acylation. Indeed a recent study has demonstrated that palmitoleic acid is a major component involved in modifying yeast PPO (Arnould et al, 1999). This is compatible with a post-translational modification of the protein resulting in the electrophoretic mobility shift which was at first attributed to the cleavage of a putative presequence.

In plants, besides the mitochondria and chloroplasts, PPO activity has been found in other subcellular locations such as the plasma membrane (Jacobs et al, 1991, Lee et al, 1993), and the endoplasmic reticulum (Retzlaff and Boger, 1996). Interestingly, the plasma membrane associated PPO activity has been found to possess properties which are different from those of the mitochondrial and chloroplastic enzymes, such as its resistance to inhibition by the diphenyl ether herbicides which are strong inhibitors of many known and characterised PPO activities (Jacobs et al, 1991) (see later in this chapter).

Effects of Various Detergents and Lipids on PPO Activity

PPOs are hydrophobic in nature and are solubilised from membranes with solutions that contain detergents such as n-octyl β -d-glucopyranoside, Tween-20, Triton X-100, and sodium cholate. In the absence of these detergents PPO tends to precipitate out of solution. The efficacy of the various detergents differs according to the PPO species solubilised. *R. sphaeroides* PPO activity was reportedly destroyed by solubilisation with Triton X-100 (Jacobs and Jacobs, 1981). A similar effect was documented with the *E. coli* and barley PPO activities in the presence of sodium cholate and Tween-20, respectively (Jacobs and Jacobs, 1984a).

Since PPOs are known to be membrane bound proteins, some workers have investigated the effects of various lipid (hydrophobic) environment on the activity of these enzymes. Jacobs and Jacobs (1984b) tested the effects of several lipids on the activities of the barley mitochondrial and etioplast PPO preparations, and showed that some of the lipids had a stimulatory effect on the activity of these enzymes. From several lipids tested, only oleic and linoleic acid had a significant stimulatory effect on these PPO activities, whilst others such as palmitic acid, phosphatidyl choline, p-ethanolamine, and caprylic acid, did not affect the activity of these proteins. At 6 mM both these lipids caused optimal stimulation of the enzymatic oxidation of both protoporphyrinogen-IX, and the nonphysiologic mesoporphyrinogen-IX. Siepker et al (1987) also showed that in the presence of 1mg/ml of oleic acid, protoporphyrinogen-IX oxidation by the purified bovine PPO was markedly stimulated by up to 66%. A

Chapter 1

similar stimulatory effect which resulted in doubling of the oxidation rate of protoporphyrinogen by a purified barley PPO preparation was observed in the presence of 1mg/ml of oleic acid, but this effect was not observed in the unpurified crude enzyme preparations from the same source (Jacobs and Jacobs, 1987). This suggested that the purification process had removed a lipid factor from the protein which could be substituted by addition of exogenous lipids. Importantly, Ferreira and Dailey (1987) demonstrated a decrease in mouse PPO activation energy and a 7-28 fold reduction in K_m when PPO was reconstituted into phospholipid vesicles. Analysis of the purified barley mitochondrial PPO for lipids, using quantitative thin layer chromatography and specific sprays, revealed that this protein was associated with various phospholipids, free fatty acids, and sterols which appeared to be important in stabilisation of the protein molecule (Jacobs et al, 1989).

It was shown that the creation of a hydrophobic environment with palmitate significantly enhanced the enzymatic oxidation of protoporphyrinogen-IX by barley and wheat etioplast PPOs (Han and Kim, 1996). Earlier Camadro et al (1994) demonstrated that the presence of 3 mM palmitate caused optimal activation of the purified yeast PPO, whilst the membrane bound PPO did not show this effect. Absence of this fatty acid in the enzyme preparation resulted in rapid loss of activity. This demonstrated that in a hydrophobic environment the protein molecule was more stable. This was in keeping with the fact that PPOs are integral membrane proteins which are buried within the lipophilic core of the membrane lipid bilayer (Deybach et al, 1985; Camadro et al, 1994).

Protoporphyrinogen Oxidase Inhibition Studies

By the time PPOs were first identified as targets for inhibition by several chemically unrelated herbicidal compounds, many of these substances were already in commercialised use as herbicides in controlling crops and weeds. The mode of action of the various herbicides was only discovered when Matringe and Scalla (1987, 1988) demonstrated that the diphenyl ether herbicide, acifluorfen (AF), caused accumulation of protoporphyrin-IX in treated plants. Importantly, they found that the accumulation of protoporphyrin was due to the inhibition of protoporphyrinogen oxidase by this herbicide (Matringe et al, 1989a & b). The abnormal accumulation of protoporphyrin-IX in tissues had been reported many years earlier in humans who were suffering from variegate porphyria, an autosomal dominant disorder which is caused by deficiency in PPO activity resulting from defective PPO proteins (Brenner and Bloomer, 1980a, Deybach et al, 1981).

An important feature of PPO inhibitors is the structural similarity of these compounds to the substrate molecule, protoporphyrinogen-IX. Whilst studies on the herbicidal PPO inhibitors showed that a bicyclic structure was the minimum structural requirement for recognition of many molecules by these enzymes it was subsequently shown that some, but not all, of the compounds with a tetrapyrrole structure were also effective inhibitors of PPOs. Other compounds such as haem and its metabolic products, biliverdin and bilirubin were also shown to be effective inhibitors of these enzymes (Poulson and Polglase, 1975, Ferreira and Dailey, 1988).

By virtue of the fact that PPOs are flavoproteins they become potential targets of general inhibitors of this class of proteins. Indeed, inhibitors of flavoproteins, such as the diphenyleneiodonium (DPI) cation and related species, bis(aryl)iodonium, have been shown to be effective inhibitors of PPOs (Arnould et al, 1997; Birchfield et al, 1998). The different categories of compounds which inhibit PPOs and the mechanisms by which they inhibit the enzyme are described below.

I. Herbicidal PPO inhibitors

For years the mode of action of many PPO inhibiting compounds remained elusive to scientists despite their widespread use as commercial herbicides in the agricultural industry. This was partly due to the fact that this was a novel mode of action for herbicides in inhibiting plant growth, and the fact that, in their investigations, scientists tended to focus their attention on the target sites of already known herbicides. Much of the research done on these compounds was therefore concentrated on plants due to the known herbicidal effect of these compounds and the vested interest of the agrochemical industry in developing more potent and highly selective compounds for commercial use in crop control.

The association of light with the development of symptoms in plants led to the implication of known photoreceptors like the carotenoids to be the targets of these compounds (Orr and Hess, 1981, Devlin et al, 1983). The photosynthetic electron transport system was also implicated in the mode of action of these herbicides (Moreland et al, 1970). Other possible modes of action included a direct involvement of these compounds in the photodynamic reaction (Orr and Hogan, 1985), mitochondrial involvement (Duke et al, 1984), and possible light-dependent phosphorylation (Pritchard et al, 1980). All these theories could not fully explain the physiological responses observed in tissues that were associated with the herbicidal treatment, and several inconsistencies in experimental findings observed by other researchers, dispelled these implications and pointed to different molecular targets (Ridley, 1983, Matringe and Scalla, 1987, Gaba et al, 1988). The pioneering studies on plant PPO activity with acifluorfen (Matringe et al, 1989a & b) and its methyl ester, methyl acifluorfen (Witkowski and Halling, 1988, 1989) produced a major breakthrough in demonstrating that the accumulation of protoporphyrin in plant tissues was due to inhibition of PPO by these compounds.

While it is implied that protoporphyrinogen-IX should accumulate as a result of PPO inhibition, it is generally accepted that there is an abnormal transient mitochondrial or chloroplast accumulation of protoporphyrinogen-IX, but that this leaks out of these organelles into the cytoplasm where it may be converted into protoporphyrin-IX in a light-dependent auto-oxidation.

The accumulation of protoporphyrin-IX as a result of PPO inhibition was positively correlated to the extent of damage in plant tissues, and it was shown that protoporphyrin-IX was the primary photodynamic molecule which was involved in the mode of action of PPO inhibiting herbicides (Duke et al, 1991a; 1994). Porphyrins are photodynamic molecules which cause formation of toxic oxygen radicals in the presence of light. This photo-sensitization may lead to formation of highly reactive oxygen radicals which cause the peroxidative destruction of membrane lipids (Hopf and Whiten, 1978; Orr and Hess, 1981; Devine et al, 1993).

Recent studies have shown that, besides the light-dependent auto-oxidation, some form of enzymatic oxidation of protoporphyrinogen occurs in the cytoplasm (Jacobs et al, 1991; Lee et al, 1993; Lee and Duke, 1994, Retzlaff and Boger, 1996). Proteins with PPO activity were demonstrated in the plasma membrane of barley cells (Jacobs et al, 1991; Lee et al, 1993; Lee and Duke, 1994) and also in the endoplasmic

reticulum of maize cells (Retzlaff and Boger, 1996), and these were shown to be relatively resistant to the inhibitory effect of the peroxidative diphenyl ether herbicides. Synthesis of haem by purified plant plasma membranes was demonstrated suggesting that there was an alternative haem biosynthetic pathway which did not involve the terminal enzymes found in the mitochondria and chloroplasts (Jacobs and Jacobs, 1995). Indeed, these investigators confirmed this in the same study when they identified ferrochelatase and iron reductase in the plasma membrane of plant cells.

Several studies in animals have shown that these compounds produce a similar effect to that of plant tissues. When male mice were fed the PPO inhibiting herbicides, oxadiazon and oxyfluorfen (1000 ppm), they developed experimental porphyria which resembled the acute phase of VP (Krijt et al, 1997). The decrease in PPO activity in herbicide treated animals resulted in elevated porphyrin content in their livers and kidneys. Approximately 80% of the total bile porphyrin content was protoporphyrin. Accumulation of porphyrins was noted in the trigeminal nerve of animals treated with oxadiazon suggesting a contribution of porphyrins to the peripheral neuropathy observed during the acute attack of porphyria. In a similar study with human progenitor cells (BFU-E/CFU-E), high concentrations of oxyfluorfen (10^{-2} M) produced a cytotoxic effect, whereas lower concentrations (10^{-4} M) only caused an inhibition of haem biosynthesis (Rio et al, 1997). Jacobs et al (1992) showed that PPO activity in chick and rat hepatocytes was inhibited in the presence of acifluorfen, methylacifluorfen, and nitrofen. The effect of porphyrins on tissues was not unexpected as photodynamic molecules have for long been regarded as herbicidal agents (Towers and Arnason, 1988).

PPO inhibiting herbicides consist of a large group of chemically diverse commercial compounds which can broadly be categorized according to their structural properties into the diphenyl ether herbicides (DPEs), phenyl heterocycles, and the heterocyclic phenylimides (figure 2.2) (Varsano et al, 1990; Matringe et al, 1992; Dayan and Duke, 1997a, b, c; Anderson et al, 1994; Reddy et al, 1997). The only reported DPE of natural origin inhibiting PPO is cyperin, which was isolated from several weed fungal pathogens (Weber and Gloer, 1988; Striele et al, 1991; Venkatasubbaiah et al, 1992).

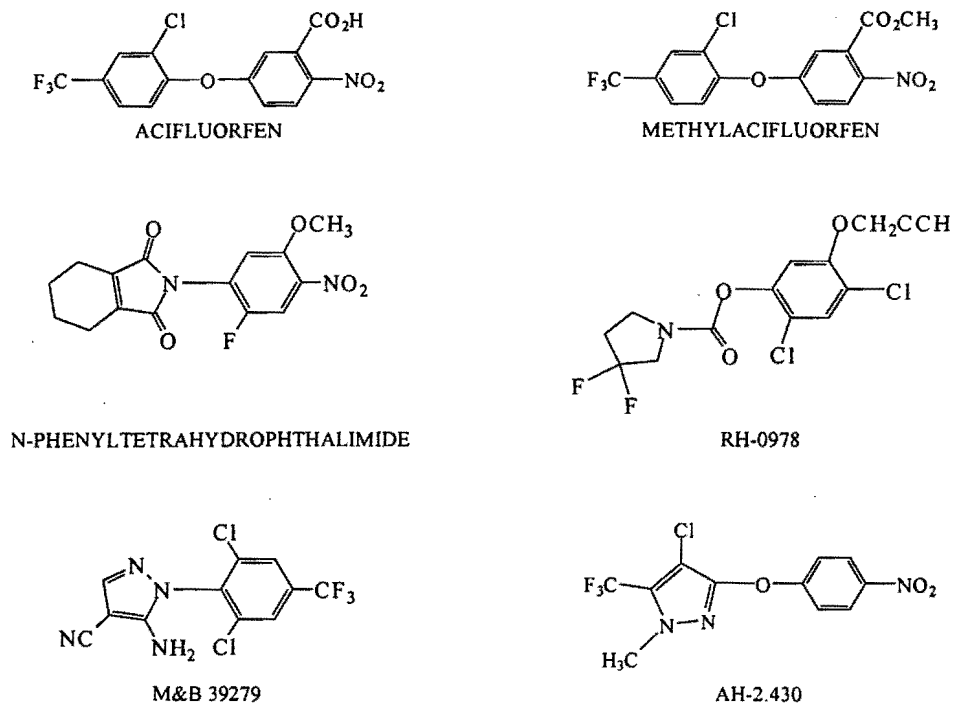


Figure 2.2 Protoporphyrinogen oxidases are inhibited by a large group of chemically diverse compounds which bear structural similarity to at least half the substrate molecule, protoporphyrinogen-IX.

An aspect which intrigued scientists about the thousands of commercially available PPO inhibiting herbicides was the fact that they were all apparently binding at the same site on the enzyme molecule. The common feature in these compounds, a bicyclic structure (figure 2.2) resembling one-half of the substrate molecule (protoporphyrinogen-IX), seems to be the basic minimum structural requirement which enables them to mimic portions of the substrate molecule and bind to the enzyme (Scalla and Matringe, 1994). Indeed, previous studies have showed that the best PPO inhibitors are those compounds that closely resemble one-half of the protoporphyrinogen-IX molecule both in terms of geometric shape and electronic characteristics (Nandihalli et al, 1992). Acifluorfen, was shown to possess the full length and one-half width of the protoporphyrinogen-IX molecule (Nandihalli et al, 1992). This study demonstrated that the molecular electronic and nuclear energies of the acifluorfen bicyclic structure matched almost half those of the protoporphyrinogen-IX tetrapyrrole structure and the dimensions of this herbicide molecule were closely matched to the bicyclic substructures of protoporphyrinogen-IX comprised of the ring AB and BC of the tetrapyrrole structure. In addition to these structural properties, this study also revealed that the enzyme inhibitory activity of PPO inhibitors was also positively related to the lipophilic nature of these compounds.

These findings have led to the successful prediction of the herbicidal activities of various compounds by quantitative structure activity relationship (QSAR) analysis (Nandihalli et al, 1992; Reddy et al, 1995; Reddy et al, 1997). The data from the QSAR studies importantly illustrated that for a compound to effectively compete with protoporphyrinogen-IX at its binding site on PPO, it must have at least two cyclic

moeities that satisfy the stereochemical properties of one-half of the substrate molecule (Nandihalli et al, 1992, 1994).

Resistance to herbicidal PPO inhibitors

In contrast to *M. xanthus* PPO (Dailey and Dailey, 1996a), the prokaryotic PPOs of *E. coli*, *B. japonicum* (Jacobs et al, 1990), and *B. subtilis* (Dailey et al, 1994a) appear to have natural resistance to inhibition by the diphenyl ethers. At 10 μ M methyl acifluorfen (AFM) the PPO activity from unextracted membranes of *E. coli* showed no significant inhibition in the presence of 1 or 5 mM DTT (Jacobs et al, 1990). Whilst the rat liver mitochondrial PPO activity was markedly inhibited by 10 μ M AFM, both the *E. coli* and *B. japonicum* were not inhibited by the same AFM concentration. These bacterial PPOs are structurally distinct from the well characterised FAD-containing PPOs thus these differences are not unexpected. At 100 μ M acifluorfen the *B. subtilis* PPO was inhibited only 18% compared to the eukaryotic enzyme which was inhibited 100% by the same inhibitor concentration (Dailey et al, 1994a). Transgenic tobacco plant cells expressing the *B. subtilis hemY* product in their cytoplasm also showed resistance to inhibition by oxyfluorfen, a competitive PPO-inhibitor (Choi et al, 1998). Subsequently, Lee et al (2000) created transgenic rice plants which were expressing the *B. subtilis* PPO gene, and these were also resistant to inhibition by oxyfluorfen. Herbicide resistant cell cultures have been identified and isolated from soybean and potato plants, but whole plants could not be regenerated from these cells (Pornprom et al, 1994).

A *Chlamydomonas reinhardtii* mutant (*rs-3*) resistant to several PPO-inhibitors such as S-23142, oxadiazon, oxifluorfen and AFE has been isolated and characterised (Kataoka et al, 1990, Sato et al, 1994). Molecular characterisation of the wild-type *C. reinhardtii* PPO showed that this protein had significant homology to the plastidial PPO isoforms of *A. thaliana* (51%) and *N. tabacum* (53%), suggesting that it was likely to be located in the chloroplasts (Randolph-Anderson et al, 1998). In this study, genetic analysis of the *C. reinhardtii rs-3* mutant PPO cDNA revealed identity with the wild-type protein, except for a G to A transition which resulted in a Val-389 to Met substitution. This residue is conserved in *A. thaliana* (Val-365), *N. tabacum* (Val-376), *B. subtilis* (Val-311) and mouse (Val-314) PPO sequences. It seems therefore that the Val to Met substitution in the *rs-3* mutant causes a conformational change at the enzyme active site which decreases its affinity for the herbicides, but does not cause apparent phenotypic changes.

Tolerance to some of the peroxidative herbicidal compounds have been observed in several plant species (Matsumoto et al, 1994; Komives and Gullner, 1994; Dayan and Duke, 1997a, b; Duke et al, 1997). Detailed investigations showed that this tolerance was not due to natural resistance of plant PPOs to these compounds. Generally, the mechanisms of plant resistance to the destructive effects of some PPO inhibitors have been identified and these include the following:

- Hindrance of the uptake and translocation of herbicide to its site of action in cells (Matsumoto et al, 1994; Komives and Gullner, 1994; Dayan et al, 1996).
- Rapid metabolic degradation of the herbicide (Frear et al, 1983).

- Natural resistance of the tissue or cells to the peroxidative effect of oxygen radicals. (Matsumoto et al, 1994; Dayan et al, 1997b).

- Resistance at the molecular site of action. Some tissues apparently develop resistance through metabolic degradation of the excess protoporphyrinogen into non-toxic compounds (Jacobs et al, 1996).

Resistance mechanisms to various PPO inhibitors are complex and are not yet fully understood. Prasad and Dailey (1995) reported the development of resistance to AF by the PPO activity of mouse erythroleukemia cells, this followed prolonged exposure of these cells to this chemical. However, this resistance to inhibition was apparently due to detoxification of acifluorfen by a cytochrome P₄₅₀ enzyme which was stimulated by the prolonged exposure to this chemical.

Recent studies have revealed that resistance to herbicides in plants could also be due to the overproduction of the target enzyme. Ichinose et al (1995) isolated and characterised photomixotrophic cultured tobacco plant cells, referred to as YZI-1S, which were resistant to inhibition by the phenyl tetrahydrophthalimide, S-23142, a competitive PPO-inhibitor. The YZI-1S cells had twice the PPO activity of wild-type cells. Molecular characterisation of the YZI-1S cells revealed that there was no difference between the plastidial and mitochondrial PPO sequences of these cells and that of wild-type cells (Watanabe et al, 1998). It was found that the mitochondrial PPO mRNA in the YZI-1S cells was ten times that of wild type cells. This led Watanabe et al (1998) to suggest that the herbicidal resistance of the YZI-1S cells was due to the overproduction of the mitochondrial PPO isoform.

In a recent study Lermontova and Grimm (2000) introduced the *A. thaliana* plastidial PPO gene into tobacco plant genome, which resulted in the overexpression of this PPO isoform in these cells. The plastids from these cells had five times the PPO activity of control wild-type cells, and they were also less susceptible to inhibition by AF. It was suggested that the overexpression of the plastidial PPO isoform was responsible for the resistance to AF.

II. Inhibition of PPOs by haem and some of its metabolic products

During the reductive metabolic breakdown of the haem molecule the methyl, propionyl and vinyl side-chains remain unchanged. Thus the intermediate haem breakdown products, viz. biliverdin and bilirubin-IX, still possess the tetrapyrrole structure and side chains found in the protoporphyrinogen-IX molecule. Therefore these compounds are potential competitive inhibitors of this enzyme, based on their structural resemblance to the substrate molecule.

Competitive inhibition of the purified mouse PPO by bilirubin ($K_i = 25 \mu\text{M}$), with respect to protoporphyrinogen-IX as substrate, was reported (Ferreira and Dailey, 1988). Both in free form, and bound to bovine serum albumin, bilirubin produced the same inhibitory effect, and $68 \mu\text{M}$ was reported to reduce the enzyme to approximately 30%. Patients with Gilbert's syndrome, which is characterised by an unconjugated hyperbilirubinaemia, reportedly have reduced PPO activity accompanied by an increase in ALA synthase activity in leukocytes (McColl et al,

1987). These enzyme disturbances are similar to those observed in patients with VP, but in the Gilbert's patients the excretion of porphyrins and their precursors was found to be normal. Previously, Evans et al (1981), also reported the increased excretion of porphyrins in a patient with hyperbilirubinaemia.

III. Inhibition of PPO by compounds that inhibit flavoproteins.

The fact that PPOs are flavoproteins has been exploited in examining new classes of inhibitors against these enzymes. The diphenyleneiodonium (DPI^+) cation and its related species have been shown to be effective inhibitors of several flavoproteins (figure 2.3) (Gatley and Sherratt, 1976, Ragan and Bloxham, 1977, Doussiere and Vignais, 1992, O'Donnell et al, 1993, Tew, 1993).

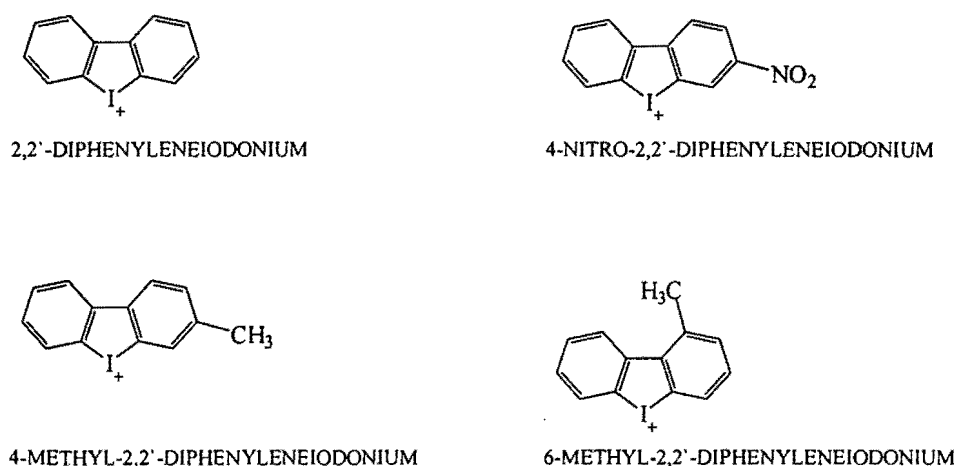


Figure 2.3 The diphenyleneiodonium cation and its derivatives are inhibitors of several flavoproteins, including PPOs. The substituted diphenyleneiodoniums were shown to be even better inhibitors of PPOs (Arnould et al, 1997).

These compounds act by removing electrons from the reduced flavin cofactors associated with flavoproteins; causing formation of phenyl radicals and covalent modification of the flavin (O'Donnell et al, 1994). A recent study on the membrane-bound yeast PPO has shown that the DPI^+ cation is an inhibitor of this enzyme with irreversible slow binding kinetics, with maximum inhibition at pH 8 (Arnould et al, 1997). A 4-nitro derivative of DPI^+ improved the PPO inhibitory effect of this compound.

Birchfield et al (1998) recently showed that 100 μM DPI^+ inhibited human PPO enzymatic activity by 48%. Furthermore, in the presence or absence of substrate (5 μM), the binding of DPI^+ to the enzyme molecule did not affect the binding of a photoaffinity radioligand, N-(5-azido-4-chloro-2-fluorophenyl)-3,4,5,6- ^3H tetrahydrophthalimide (^3H AzTHP). Previous work showed that tritiated

tetrahydrophthalimide ($[^3\text{H}]\text{THP}$) undergoes rapid, specific, saturable, and reversible binding to the substrate/herbicide binding site on solubilized mouse mitochondrial PPO (Birchfield and Casida, 1996). Both acifluorfen and the more potent THP, which compete with protoporphyrinogen for binding at the catalytic site, were shown to be effective competitive inhibitors of $[^3\text{H}]\text{AzTHP}$ binding (Birchfield et al, 1998), indicating that these compounds were sharing the same or overlapping binding site on the enzyme molecule. These findings demonstrated that DPI was not sharing the same binding site occupied by $[^3\text{H}]\text{AzTHP}$, acifluorfen and THP on the enzyme molecule. This suggests that the FAD binding site is apparently distant from the substrate/herbicide binding site on the human PPO molecule.

Quinacrine (16 mM), a known inhibitor of FAD-containing enzymes such as the monoamine oxidases, was shown to be an effective inhibitor of the *B. subtilis* hemY protein (Hansson et al, 1997) and suggested that the enzyme was a FAD flavoprotein.

PPO Immunological Studies

Very few immunological investigations appear to have been done on PPOs. This has partly been due to the fact that this protein has proved very difficult to purify over the years. Siepker et al (1987) reported the raising of antibodies against the homogeneously purified bovine protein. On Western blotting, this antibody specifically recognized the 57 000 Da PPO protein, and another 53 000 Da protein which co-purified with PPO. They reported 80% cross-reactivity of the purified IgG fraction of this antibody with bovine ferrochelatase. The purified PPO also cross-reacted with antibodies raised against purified bovine ferrochelatase, but the 53 000 Da protein was not recognized.

Subsequent to the identification, cloning and purification of the human PPO, purified human IgG antibodies cross-reacted with purified recombinant mouse PPO (Taketani et al, 1995a). As these two proteins share 86 - 89% sequence identity (Taketani et al, 1995a, Dailey et al, 1995), this is not surprising. Antibodies raised separately against the plastidal and mitochondrial isoforms of the tobacco plant PPO showed no cross-reactivity (Lermontova et al, 1997). This lack of cross-reactivity is explained by the 27% sequence homology between these PPOs, even though they are from the same species, and confirms the different ancestral origins of these two organelles.

An antibody raised against yeast PPO was used as a tool in many of the studies on yeast PPO carried out by Camadro et al. (1994, 1996).

The Cloning and Identification of PPO Genes

In their study of the genes involved in haem biosynthesis in the aerobic bacterium, *B. subtilis*, Hansson and Hederstedt (1992) reported the identification of an open reading frame (*hemY*) which was suggested to be involved in the oxidation of protoporphyrinogen-IX. Initially the protein encoded by this gene was thought to possess either protoporphyrinogen-IX or both protoporphyrinogen-IX and coproporphyrinogen-III oxidising activities. Interestingly, Dailey et al (1994a) found that this reported protein had some sequence similarity to several peptide sequences

of the mammalian PPO gene which they were cloning at that time (personal communication, P. Meissner). Thus Dailey et al (1994a) cloned and expressed the *B. subtilis* *hemY* gene in *E. coli* and showed that it encoded a monomeric protein with a predicted M_r of 51 200 Da which had PPO activity. This provided conclusive evidence that this protein was PPO and they suggested that the gene should be designated *hemG*. A striking feature of this protein was its ability to oxidise both protoporphyrinogen-IX and coproporphyrinogen-III. Subsequent characterisation revealed that the product from coproporphyrinogen-III oxidation was coproporphyrin-III, and not protoporphyrinogen-IX which is formed during the oxidative decarboxylation of this compound by coproporphyrinogen-III oxidase (Hansson and Hederstedt, 1994b; Hansson et al, 1997).

Shortly after the identification of the *B. subtilis* enzyme by Hansson et al (1992), another prokaryotic PPO was isolated from the facultative anaerobic Gram negative, *E. coli* K12 strain (Sasarman et al, 1993). The *E. coli* *hemG* gene was sequenced and shown to be encoding a 21 200 Da (181 amino acid residues) protein which complemented a PPO-deficient mutant of *E. coli*. Later the *hemG* gene from a similar *E. coli* strain was cloned and sequenced by Nishimura et al (1995a) and shown to be encoding the same PPO protein identified previously by Sasarman et al (1993).

Nakayashiki et al (1995) cloned and sequenced a different *E. coli* gene, termed *hemK*, which they thought was involved in oxidation of protoporphyrinogen-IX, but its function was not fully elucidated. They found that this gene and its protein product (225 amino acid residues) had no significant sequence homology to any reported PPO gene or protein sequence, but was rather more similar to the N-terminal region of a *Salmonella typhimurium* gene, previously sequenced (Elliot, 1989). Phenotypic analysis and activity assay of a yeast *hemK* analog protein which was identified by Le Guen et al (1999) revealed that the isolated protein was not involved in protoporphyrinogen-IX oxidation. Sequence analysis with related proteins showed a consensus motif for S-adenosyl-methionine-dependent methyltransferase. Complementation experiments with PPO-deficient yeast strains in which this protein was overproduced showed no restoration of wild-type phenotypes. The putative *hemK* is probably a subunit of another protein that has nothing to do with PPO activity.

A PPO gene from the aerobic Gram negative bacterium, *M. xanthus*, was cloned and sequenced by Dailey and Dailey (1996a). The expressed protein was purified and partially characterised. It has a predicted subunit M_r of 49 387 Da, (471 amino acid residues). Despite the fact that *M. xanthus* and *B. subtilis* are both aerobic bacteria comparison of the amino acid sequences of their PPO sequences revealed only 23% identity between them. Most of the sequence homology was found in the N-terminal sequence of these proteins containing the dinucleotide binding motif (Dailey et al, 1994a; Dailey and Dailey, 1996a). As more prokaryotic PPOs are identified and sequenced it appears that they share very little homology between themselves suggesting that the mechanisms by which they oxidise protoporphyrinogen-IX may differ. Indeed, this is so as it has been shown that these prokaryotes can live under aerobic (strict aerobes) or anaerobic (obligate anaerobes) conditions, or both (facultative anaerobes).

Hem14, the structural gene for yeast PPO was isolated and identified by Camadro and Labbe (1996) by means of functional complementation of a *hem14-1* PPO-deficient yeast mutant. This gene encodes a 59 665 Da protein (539 amino acid residues), with an N-terminal sequence which these authors claimed to be consistent with leader peptides of proteins targeted to the mitochondria. Indeed, in previous work they had shown purified yeast PPO to be synthesized as a high molecular weight precursor protein (58 000 Da) that was apparently converted *in vivo* through the cleavage of the leader sequence into the mature smaller (55 000 Da) membrane bound protein (Camadro et al, 1994). More recently, however, the apparent difference in molecular sizes has been ascribed to acylation of the protein, which is known to affect electrophoretic mobility rather than cleavage of a mitochondria-targeting leader sequence (Arnould et al 1999).

In a separate investigation, Glerum et al (1996) also cloned and identified the yeast *hem14* gene encoding a 59 700 Da PPO protein with similar properties to the apparent precursor form of this enzyme reported by Camadro et al (1994). These workers reported 27% identity and 51% similarity of this protein to the human PPO protein sequence.

There is some information on PPO genes and proteins from plants. Narita et al (1996) reported the first identification of a plant cDNA gene encoding PPO from *A. thaliana*. The gene was identified by functional complementation of a *hemG* mutant *E. coli* strain. Molecular cloning and characterisation of this gene revealed that it encoded a protein of 537 amino acid residues with a calculated molecular mass of 57 700 Da. The amino acid sequence of this PPO shared approximately 27% homology with *B. subtilis*, mouse, and human PPOs, and this was mainly in the region of the dinucleotide binding domain. The deduced PPO protein sequence appeared to have an N-terminal leader sequence characteristic of mitochondrial targeting peptides.

Lermontova et al (1997) reported the cloning of two different full length cDNA gene sequences from tobacco plant which encoded plastidal and mitochondrial isoforms of PPO. These two PPO isoforms from the same plant shared 27% amino acid sequence identity. The plastidal isoform was synthesized as a 59 138 Da protein (548 amino acid residues) with a putative 50 amino acid residues leader sequence, and was shown to be processed into a 53 000 Da mature protein when translocated into chloroplasts. Interestingly, this PPO isoform was similar in size to the yeast and *A. thaliana* proteins described above, and it also has a membrane targeting leader sequence like these other PPOs. Sequence analysis showed that this protein has 71% identity to the *A. thaliana* protein sequence. The mitochondrial PPO isoform had a calculated molecular mass of 55 407 Da (504 amino acid residues) and was shown to be targeted and imported into the mitochondria without any detectable size reduction.

Other plastidal and mitochondrial PPO isoforms have been cloned and characterised from the potato plant (Johnston et al, 1998). These PPOs were cloned from a cDNA library obtained from four week old potato leaf tissue. Primers used in isolating the clones were designed from homologous regions of *A. thaliana* (Narita et al, 1996) and maize (Ward patent number WO 95/34659, 1995) PPO sequences. The full-length cDNA of the plastidal PPO isoform encodes a 60 426 Da (557 amino acid residues) protein with 72, 89 and 78 percent similarities to *A. thaliana*, tobacco and maize

plastidal PPO isoforms, respectively. The mitochondrial partial sequence had similarities of 62, 92 and 55% to *A. thaliana*, tobacco and maize mitochondrial isoforms, respectively. The translocation of these PPO isoforms to their respective organelle destinations was identified by sequence homology to previously known proteins destined for the same locations. It seems that different plant PPO isoforms destined for a particular organelle apparently share a common ancestry as suggested by the high sequence homology or the sharing of common epitopes which can be demonstrated by immunological cross-reactivity. The lack of homology between plastidal and mitochondrial protein isoforms from the same species further illustrates the point that these two organelles have different evolutionary origins.

The cloning, sequencing and expression of the prokaryotic PPOs from *B. subtilis* (Hansson and Hederstedt, 1992, 1994b; Dailey et al, 1994a) and *E. coli* (Sasarman et al, 1993) facilitated the discovery and identification of the mammalian genes encoding mouse (Dailey et al, 1995; Taketani et al, 1995a) and human PPOs (Nishimura et al, 1995b; Dailey and Dailey, 1996b, 1997a, b; Taketani et al, 1995b; Roberts et al, 1995b). Genomic DNA fragments containing the whole coding sequence for human PPO (1431 base pairs) have been cloned (Roberts et al, 1995b). This gene encodes a 51 000 Da (477 amino acid residues) protein which exists as an approximately 100 000 Da homodimer (Dailey and Dailey, 1996b).

The human PPO gene was mapped by fluorescence *in situ* hybridisation to chromosome 1q22-q23 of the human genome (Taketani et al, 1995b; Roberts et al, 1995b). This was in contrast to the previous localisation of the VP locus by linkage and haplotype analysis to chromosome 14q32 (Bissbort et al, 1988). Further evidence against the linkage between the VP locus and microsatellite markers at chromosome 14q32 came from microsatellite DNA marker studies of this region (Warnich et al, 1996b). The human PPO gene has 13 exons spanning approximately 5 kb, and the intron/exons boundaries have been defined (Roberts et al, 1995b; Taketani et al, 1995b). Some disagreements exist on the lengths of introns 4, 7, and 9 (Puy et al, 1996). Northern blot analysis from a variety of tissues suggest a single mRNA transcript for human PPO of approximately 1.8 kb in length (Nishimura et al, 1995b; Dailey and Dailey, 1996b). These transcripts contain an approximately 300 bp long 5' untranslated region (UTR) and a short 3'-UTR. Researchers have identified the start and termination codons, as well as a consensus polyadenylation signal and polyadenylation site downstream from the termination site (Taketani et al, 1995b; Dailey and Dailey, 1996b; Puy et al, 1996). The mouse PPO was shown to be similar to the human enzyme in terms of its size, 51 000 Da (477 amino acid residues), and homology - the two proteins share 89% amino acid sequence identity and both lack typical membrane targeting signals despite their localisation to the mitochondrial inner membrane (Dailey et al, 1995; Taketani et al, 1995b).

General Comments

Whilst auto-oxidation of protoporphyrinogen to protoporphyrin can occur in the presence of oxygen; the reducing environment inside cells where this reaction takes place (mitochondria and chloroplasts) requires catalysis by PPO. During the aerobic reaction molecular oxygen is utilised as the terminal electron acceptor. In prokaryotes

several alternative compounds such as nitrate, and fumarate are utilised as the terminal electron acceptors in this reaction. It would be interesting to see if different PPO isoforms based on their oxygen requirements exist in these organisms, but thus far such isoenzymes have not been unequivocally identified. Oxygen-dependent (*hemF*) and oxygen-independent (*hemN*) isoforms of coproporphyrinogen oxidase have been identified in some facultative prokaryotes such as *E. coli* (Troup et al, 1994, 1995).

An interesting feature of PPOs is their inhibition by thousands of chemically diverse compounds. Whilst eukaryotic PPOs are strongly inhibited by bicyclic compounds, these appear to have very little effect on the activity of prokaryotic enzymes such as those of *E. coli*, *B. japonicum* (Jacobs et al, 1990) and *B. subtilis* (Dailey et al, 1994a). However, the behaviour of the *M. xanthus* PPO rules this out as a general prokaryotic property, because it was shown to be strongly inhibited by the diphenyl ethers (Dailey and Dailey, 1996a).

Investigation of several PPO genes by molecular biology methods has revealed that many of these proteins lack any identifiable membrane targeting signals despite the fact that it has been established that these are intrinsic inner mitochondrial membrane proteins. However, the N-terminal leader sequence required for membrane targeting and translocation has been suggested in few of these proteins (Camadro et al, 1994; Narita et al, 1996; Lermontova et al, 1997). It appears therefore that many of these proteins possess internal targeting signals that have not yet been definitively identified.

Very interesting information is beginning to emerge as more PPO genes are identified and characterised. Certainly, the application of molecular biology approaches in the study of these proteins will facilitate their characterisation.

Chapter Three

Development of this Thesis

Context in Which this Work was Performed

Protoporphyrinogen oxidase (PPO) catalyses the penultimate step in haem biosynthesis that is, the six electron oxidation of protoporphyrinogen-IX into protoporphyrin-IX (Kappas et al, 1989, Dailey, 1990). A genetic defect in human PPO is responsible for VP, an autosomal dominant disorder characterised by skin photosensitivity and propensity towards acute neurovisceral crises (Kappas et al, 1989). In South Africa this disease has an exceptionally high frequency which is due to a founder gene effect (Dean, 1971, Eales et al, 1980, Day, 1986, Meissner et al, 1986, 1996, Warnich et al, 1996a). Thus it is natural that VP and the defective enzyme responsible for this condition is of great interest to the Lennox Eales Porphyria Laboratories of the University of Cape Town from both an academic and pragmatic point of view.

Previous Studies

Previous studies, based on investigations carried out on crude unpurified and sometimes partially purified PPOs using standard chromatographic procedures, revealed differences among these proteins from different species (Poulson and Polglase, 1975, Poulson, 1976, Jacobs and Jacobs, 1981, 1987, Klemm and Barton, 1987, Dailey and Karr, 1987, Siepker et al, 1987, Ferreira and Dailey, 1988, Jacobs et al, 1989, Proulx and Dailey, 1992). These studies indicated that PPOs may be different in terms of their molecular weights, subunit composition, cofactors, kinetic properties and terminal electron acceptors involved in the oxidation of protoporphyrinogen-IX to protoporphyrin-IX. The fact that these PPOs were characterised in the presence of other proteins which may influence their behaviour in one way or the other resulted in lack of information on the biochemical behaviour and molecular nature of these proteins. Hence, the need for protein purity in order to carry out reliable characterisation.

The Project

Thus, at the beginning of this project little information was available on the kinetic and biophysical properties of isolated PPOs due to difficulties associated with purification of these proteins from natural sources, using standard chromatographic techniques. This contributed in part to PPO being the last of the haem biosynthetic enzymes to be characterised both at the protein and gene levels. Detailed characterisation of PPO therefore still remained a relevant challenge. Hence this study (and others conducted elsewhere).

The application of molecular biology techniques in the study of proteins has facilitated their characterisation, leading to improved purification strategies of

recombinant proteins. In this work we demonstrate a greatly facilitated characterisation of various forms of PPO based on the availability of recombinant PPO expression systems.

Consequently, this thesis describes work undertaken towards the optimisation of expression and purification of recombinant PPOs from three species (*B. subtilis*, *M. xanthus* and human) to apparent homogeneity, using the 6X His tag system which is based on metal chelate affinity chromatography (Chapter 4). This allowed the description of the kinetic behaviour of the purified PPOs and determination of their inhibitor profiles (Chapters 5 and 6). Sequence information (nucleotide and amino acid) available from publicly accessible databases has also proved very useful in analysing the relationship amongst PPOs from several diverse species. Hence a chapter describing a phylogenetic analysis of these proteins from different species as obtained from the GenbankTM and TIGR databases is thus included (Chapter 7).

A further focus of this project is based on our ability to produce a monospecific antibody to human PPO. This has enabled the immunocharacterisation of the human enzyme in tissue sections by light microscopy. Finally, using a modification of standard immunogold labelling techniques we have succeeded in viewing PPO at an intracellular level using electron microscopy. This led to the demonstration of PPO expression in different human tissues/organs, and its subcellular localisation in human hepatocytes (Chapter 8).

Chapter Four

Expression and Purification of the Recombinant Protoporphyrinogen Oxidases of *Bacillus subtilis*, *Myxococcus xanthus* and Human

Introduction

In the study of PPO, a difficulty that workers encountered initially was the apparent low expression of PPO in readily available tissue or culture material. Thus large amounts of fresh tissue/culture were desirable. Secondly, in many cases there appeared to be a rapid loss of PPO activity, making monitoring of PPO purification procedures arduous. Finally, some inherent properties of PPO (intrinsic membrane localisation, cofactor requirement) rendered isolation of the protein biotechnically difficult.

The publication of the *B. subtilis* *hemY* gene sequence was seminal in that it provided information on a putative PPO gene (Hansson and Hederstedt, 1992). The definitive identification of this as the *B. subtilis* PPO (*hemG*) gene and the development of a viable expression system of the soluble form of the *B. subtilis* PPO from the pBTac1 expression vector in *E. coli* (Dailey et al, 1994a) was a major breakthrough. Because of our group's focus on VP and PPO, this also resulted in a collaborative (ongoing) study between Professor Harry Dailey (University of Georgia, USA) and our laboratory.

This chapter describes the optimisation of the expression of the recombinant *B. subtilis* PPO with a 6 histidine (6His) tag at its N-terminus using an isopropyl-1-thio- β -D-galactoside (IPTG) inducible pTrcHis vector. The 6His tag renders the protein susceptible to immobilised metal chelate affinity chromatography by virtue of the binding ability of histidine to divalent metal ions such as nickel. This system enabled establishment of a single step purification of homogeneous recombinant PPO and was adapted for the purification of the *M. xanthus* and human PPOs.

The methodology used is outlined as necessary but further detail is supplied in Appendix 1.

Objectives

- To express the previously cloned PPO genes of *B. subtilis*, *M. xanthus* and human in *E. coli* cells.
- To purify these recombinant PPOs to apparent homogeneity using metal chelate affinity chromatography.

Methods and Materials

Cloning of the *B. subtilis* PPO gene

The cloning of the *B. subtilis hemY* gene, was carried out in Professor Dailey's laboratory at the University of Georgia, Athens (USA) and was provided as a gift to our laboratory. The methodology used is described below:

A fragment containing the full-length coding region of the *B. subtilis* PPO gene was generated by polymerase chain reaction (PCR), using the *B. subtilis hemY* gene sequence previously cloned into the pBtac1 vector (Dailey et al, 1994a) as a template. This fragment was cloned into a pTrcHis B expression vector (Invitrogen, Carlsbad, California, USA). A 5' *Bam*HI site and a 3' *Hind*III site were included in the primers for cloning purposes. Specifically, the sense primer was 5'-CGC **GGA TCC** ATG AGT GAC GGC AAA AAA-3'. The *Bam*HI site is in bold, and the starting codon of the *B. subtilis* PPO sequence is underlined. The antisense primer used was 5'-TAACCGGCAAGCT TCA GCT GAA TAA ATA GGT AAG-3' (Dailey et al, 1994a). The PCR contained 1 μ M final concentration of each primer, 50 ng template, 2 mM MgCl₂, 200 μ M dNTPs and 5 U *Taq* polymerase (Promega, Madison, Wisconsin, USA). The PCR program was as follows: 3 min at 95⁰C, [1 min at 95⁰C, 1 min at 56⁰C, 2 min at 72⁰C] X 30 cycles, and a final extension time of 7 min at 72⁰C. A single PCR product of about 1400 bp was obtained. The product was purified on an agarose gel, excised and extracted from the gel by the Magic PCR preparative procedure (Promega). The resulting cDNA fragment was digested with *Bam*HI and *Hind*III and cloned into pTrcHis B, which had also been digested with *Bam*HI and *Hind*III. The resulting recombinant plasmid was named pBsubtPPO-X.

Transformation, Expression and Purification of the Recombinant *B. subtilis* PPO

Transformation of the PPO-containing plasmid into E. coli

Work in our laboratories commenced with the transformation of the recombinant *B. subtilis* PPO into competent *E. coli* JM109 cells for expression purposes.

Competent *E. coli* JM109 cells (Promega) were transformed with the pBsubtPPO-X plasmid. 500 μ l of an overnight *E. coli* JM109 culture grown in Luria Bertani (LB)

broth (1% Tryptone, 0.5% Yeast extract, 0.5% NaCl) was transferred into 50 ml of sterile LB broth in a 500 ml flask and incubated at 37°C with shaking until an OD₆₀₀ of 0.5-0.6 was reached (mid log phase). The cells were then harvested by centrifugation at 2000 G for 10 min at 4°C. After draining off the supernatant the cell pellet was gently resuspended in 300 µl of ice cold transformation buffer (1% Tryptone, 0.5% yeast extract, 0.5% NaCl, 20 mM MgCl₂, 10% polyethylene glycol, 5% dimethyl sulfoxide) and left on ice for 30 min. 100 µl of the suspension was gently mixed with 1 µl (~30 ng) of pBsubtPPO-X DNA in an Eppendorf tube and left on ice for 45 min. This mixture was then put on a uniform heating block prewarmed to 42°C and left for 2 min, and then transferred immediately to an ice bath for 1.5 min. 900 µl of sterile LB broth was added and this transformation mixture was incubated for 1 h at 37°C with shaking. Different volumes (100 µl, 200 µl and 500 µl) of the transformed culture were plated onto LB plates containing 100 mg/ml ampicillin and incubated overnight at 37°C. Single colonies of the transformed cells were isolated from the plates and inoculated into 100 ml of LB broth containing 100 mg/ml ampicillin and grown overnight at 37°C, and the best PPO-expressing colony (based on PPO activity assays) was used to prepare 1 ml aliquot stocks (70% culture + 30% sterile glycerol) were stored at -80°C for future use.

Control *E. coli* JM109 cells (lacking ampicillin resistance) from an overnight culture were also plated onto ampicillin (100 mg/ml) containing LB plates to test the ability of the antibiotic concentration to suppress growth of non-resistant cells.

Expression of the recombinant PPO

For the expression of the recombinant *B. subtilis* PPO, 100 µl of transformed *E. coli* JM109 cells containing pBsubtPPO-X were inoculated into 10 ml LB broth containing 100 µg/ml ampicillin and incubated overnight at 37°C with shaking in an orbital shaker incubator. 100 µl of the overnight culture was inoculated into 1 L of LB broth containing 100 µg/ml ampicillin and the cells were grown to mid-log phase (OD₆₀₀ of 0.5-0.7) at 37°C. 1 mM IPTG was added to the culture for the induction of recombinant *B. subtilis* PPO expression, and growth was continued for 4 h. A similar control culture was prepared in 1 L of LB broth without ampicillin using *E. coli* JM109 cells that were not transformed. Cells from the cultures were harvested by centrifugation at 12000 G for 30 min at 4°C, and resuspended in 3 ml of cold assay buffer (100 mM Tris, 3 mM DTT, 1 mM EDTA, 0.1% Tween-20, pH 8.1) and kept on ice. The cells were sonicated for 3X 30 sec cycles on ice. Samples from both the transformed (pBsubtPPO-X) and non-transformed (control) lysates were assayed for PPO activity according to the method of Meissner et al (1986). Essentially, the method is based on the measurement of the constant velocity formation of protoporphyrin-IX from protoporphyrinogen-IX under saturating substrate conditions using fluorimetry.

Purification

Numerous initial attempts to purify PPO to homogeneity under native (non-denaturing) conditions proved only partially successful. Therefore we approached the

problem by developing a purification procedure using denaturing conditions, followed by renaturation. This approach was validated in that the kinetic parameters obtained using this material were not significantly different from those exhibited by semi-purified (non-denatured) material.

Purification of recombinant B. subtilis PPO using denaturing conditions

Cells were harvested by centrifugation at 4000 G for 30 min at 4°C. The pellet was resuspended in a denaturing solubilisation buffer (6 M GuHCl, 0.1 M Na-phosphate, 0.01 M Tris, pH 8.0) at 5 ml per g wet weight, and stirred at room temperature for 1 h. The lysate was centrifuged at 105000 G for 30 min, and the PPO-containing supernatant collected.

The clear supernatant containing recombinant *B. subtilis* PPO was loaded onto a Ni-NTA agarose column pre-equilibrated with the solubilisation buffer. After an initial wash with 20 ml of solubilisation buffer (6 M GuHCl, 0.1 M Na-phosphate, 0.01 M Tris, pH 8.0), the column was washed with 20 ml of a second denaturing buffer (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris, pH 8.0), which was followed by another wash with 20 ml of the same buffer with pH adjusted to 6.3. PPO was eluted with 0.25 M imidazole in the pH 6.3 buffer and collected in 1 ml fractions. PMSF (1 µg/ml) was added in all buffers immediately before use, to prevent protease degradation. The entire procedure was performed at room temperature and PPO activity monitored throughout.

Renaturation of the protein was achieved by dilution of at least 50 times with the assay buffer (100 mM Tris, 1 mM EDTA, 3 mM DTT, 0.1% Tween-20, pH 8.7), in the presence of 5 µM FAD, and incubation for at least 30 min at 37°C prior to activity measurements. (Reactivation whilst the protein was immobilised on the Ni-NTA column prior to elution proved unsuccessful when renaturation was initially tried by using a 6 - 1 M urea gradient in 0.5 M NaCl, 20% glycerol, 0.01 M TrisHCl, pH 7.4.)

The purity of the PPO was assessed using SDS-PAGE.

Partial purification of recombinant B. subtilis PPO using non-denaturing (native) conditions

Cells from 1 L of transformed *E. coli* JM109 culture (with pBsubtPPO-X) were resuspended in 30 ml of sonication buffer (0.05 M Na-phosphate, 0.3 M NaCl, 1% Tween-20, pH 8.0). PPO was released from the cells by sonication for 4X 30 sec, on ice. The PPO-containing supernatant was collected after centrifugation of the lysate at 105000 G for 30 min at 4°C. The supernatant was loaded onto a Ni-NTA agarose column pre-equilibrated with sonication buffer as above except that there was 0.2% Tween-20. After loading, the column was washed with equilibration buffer (20 ml). After a wash with 0.05 M Na-phosphate, 0.01 M TrisHCl, 0.3 M NaCl, 10% glycerol, 0.2% Tween-20, pH 6.3, bound protein was eluted from the column with a 0-0.1 M imidazole gradient in this buffer. PMSF (1 µg/ml) was added to all buffers.

The purity of the PPO was assessed using SDS-PAGE.

Cloning, Transformation and Expression of the *M. xanthus* and Human PPO genes

M. xanthus PPO

The construction of the recombinant plasmid containing the *M. xanthus* PPO gene was performed in Prof. Dailey's laboratories using the plasmid pLJS43. Details are published (Dailey and Dailey, 1996a) and the protocol resulted in an engineered expression vector (pMx-PPO) essentially similar to the pBsubtPPO-X described above which encoded a protein that contained a 6X His tag. However, rather than cloning into the pBTac1 vector, the pTF20E derivative of this vector was used in which there was an optimally spaced ribosomal binding site T7 enhancer and an ATG start site placed immediately downstream from the promoter. This enhanced protein expression and thus greatly facilitated purification. The pMx-PPO was also a gift to our laboratory.

We transformed *E. coli* JM109 cells with this recombinant plasmid essentially as described for the *B. subtilis* gene. The JM109 cells were, however, rendered competent using the CaCl₂ method (see Appendix 1 for details).

The expression system as described for the *B. subtilis* protein proved entirely suitable for the expression of the *M. xanthus* PPO except that no IPTG induction was necessary and no initial 10 ml overnight culture appeared necessary. Thus we inoculated 1 L LB broth with 100 µl of glycerol stock of the plasmid and cultured for 24 h prior to using for purification.

Human PPO

The construction of the recombinant plasmid containing the human PPO gene was performed in Prof. Dailey's laboratories (Dailey and Dailey, 1996b). The 6X His tag was placed upstream from the ATG start site as insertion of this tag immediately adjacent to the ATG start codon resulted in poor binding to the metal affinity column. A nine amino acid spacer was added to the front of the protein. The engineered cDNA was inserted into the unique *Nhe*I site of the pTrcHis B vector and was named pHPPO-X. Like the other two PPO's this was also a gift to our laboratory.

Transformation and expression of *E. coli* cells with the HPPO-X plasmid was as described for the *M. xanthus* gene.

Purification of *M. xanthus* PPO

The cultured, PPO-expressing cells were harvested by centrifugation at 4000 G for 30 min at 4°C. The pellet was resuspended in 30 ml of sonication buffer (0.02 M Tris-HCl, 0.1 M NaCl, 1% Tween-20, pH 8.0) and sonicated as above and centrifuged at 105000 G for 30 min. The subsequent purification, although similar in principle to that of the *B. subtilis* purification, differed and is described here. We used "Talon", which is a Co-based immobilised metal-affinity chromatography resin rather than the

from the above spin, loaded. The column was washed with 10 ml of 0.02 M Tris-HCl, 0.1 M NaCl, 0.5% Tween-20, 10% glycerol, pH 6.3, followed by 10 ml of this buffer but with 0.05 M phosphate replacing the Tris-HCl. At this stage the column was washed with 10 ml of this phosphate buffer containing 25 mM imidazole in order to remove non-specifically bound proteins. Elution of PPO was in 100 mM imidazole in the phosphate buffer. 1 μ g/ml PMSF was added to all buffers as a protease inhibitor.

Purification of Human PPO

n-octyl- β -D-glucopyranoside was the detergent of choice for the solubilisation of the human PPO and replaced Tween-20 in all buffers. Tween-20 proved unsuitable in initial purification attempts. The subsequent purification of the recombinant human PPO was on Talon resin equilibrated in 0.02 M Tris-HCl, 0.1 M NaCl, 1% *n*-octyl- β -D-glucopyranoside, pH 8.0. After loading under gravity the column was washed with 20 ml of equilibration buffer prior to a 10ml wash in the same buffer containing 0.025M imidazole followed by elution of bound PPO with 0.15 M imidazole in the same buffer.

All the purification schemes were carried out at room temperature. The *M. xanthus* and human PPO protein fractions were immediately stored at 4⁰C after elution, and the *B. subtilis* PPO (which was in denaturing buffers) was retained at room temperature.

Results and Discussion

B. subtilis PPO

Expression

The sonicate from transformed cells containing the recombinant plasmid which expresses *B. subtilis* PPO showed a significantly higher rate of protoporphyrinogen-IX oxidation. There was an approximate 17-fold increase in PPO activity in the transformed cells when expressed as nmol of protoporphyrinogen oxidised/h/mg protein (figure 4.1). This indicated successful transformation of the cells and retention of the recombinant plasmid, pBsubtPPO-X, which was expressing *B. subtilis* PPO.

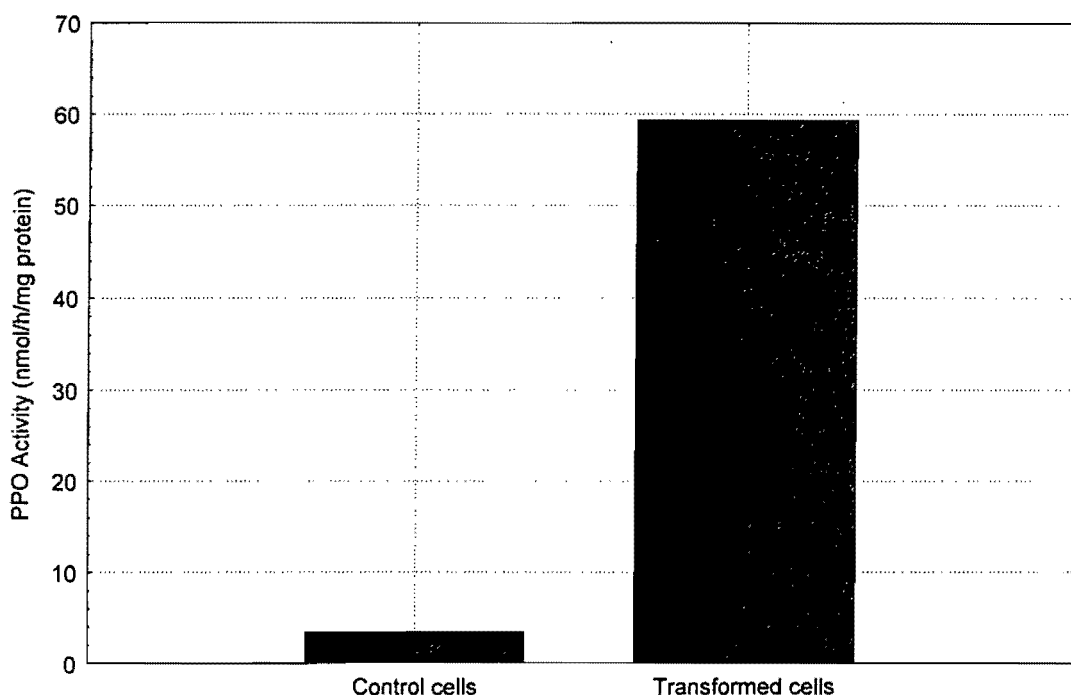


Figure 4.1: *B. subtilis* PPO activity per mg protein from transformed *E. coli* cells was approximately 17 times that of control cells.

Purification

Purification of the *B. subtilis* PPO using non-denaturing (native) conditions yielded partially purified PPO with M_r of approximately 56000 Da, which co-eluted with low concentrations of some low molecular weight contaminants (figure 4.5). As mentioned in the "Methodology", these contaminants were not removed in spite of numerous attempts of varying purification conditions. A few of these bands may have represented breakdown products.

Table 4.1 shows that the purification of the protein under these non-denaturing (native) conditions from 1 L of *E. coli* culture, gave a 68% yield and an approximately 10-fold increase in activity per mg of protein.

In contrast, the purification scheme using denaturing conditions yielded a single protein of 56 000 Da (figure 4.2) and a 78.5% purification of PPO from the *E. coli* lysate with a 31-fold increase in specific activity (activity per mg of protein) for the renatured protein (table 4.2). Approximately 4 mg of PPO were obtained from 1 L of culture.

Table 4.1: Partial purification of *B. subtilis* PPO by Ni-NTA metal chelate affinity chromatography using non-denaturing conditions.

	Fractn Vol.	Total Prot.	PPO Act.	Total Act.	Act. / mg (apparent)	% Yield (apparent)
	(ml)	(mg)	(nmol/h/ml)	(nmol/h/ml)	(nmol/h/mg)	
Load	28	114.5	195.8	5482.4	47.8	100
Eluate	10	7.6	374.6	3746.0	492.9	68.3

Table 4.2: Purification of *B. subtilis* PPO by Ni-NTA metal chelate affinity chromatography using denaturing conditions.

	Fractn Vol.	Total Prot.	PPO Act.	Total Act.	Act. / mg	% Yield
	(ml)	(mg)	(nmol/h/ml)	(nmol/h/ml)	(nmol/h/mg)	
Load	15	150	56	840	5.6	100
Eluate	1	3.8	659.6	659.6	173.37	78.5

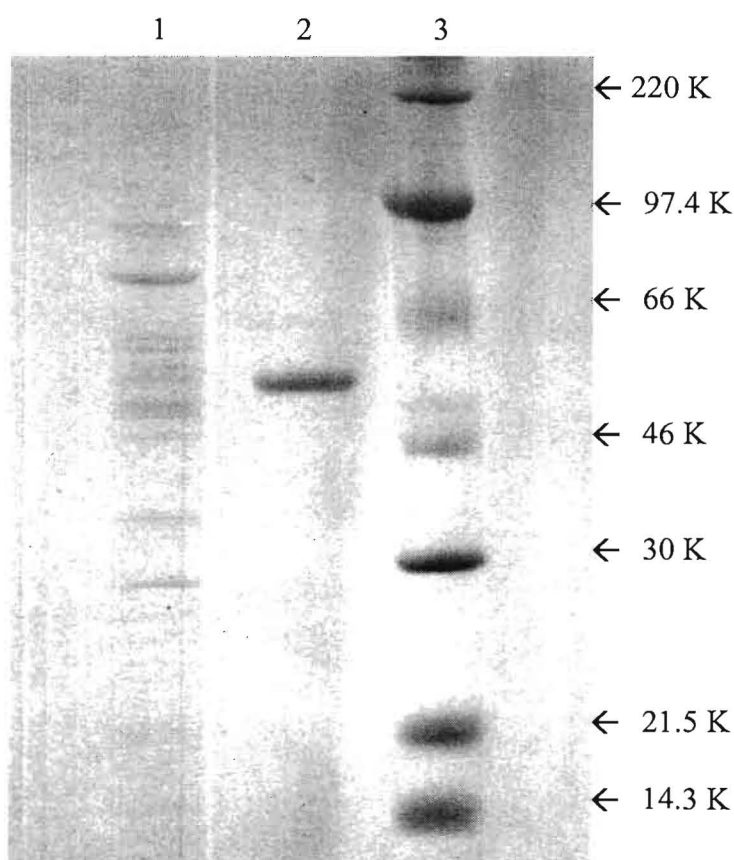


Figure 4.2: SDS PAGE (7.5-17.5%) showing purification of the *B. subtilis* PPO using denaturing conditions.

Lane 1 - supernatant from *E. coli* cells, expressing *B. subtilis* PPO, which were lysed with 6 M GuHCl buffer showing a wide spectrum of proteins.

Lane 2 - purified *B. subtilis* PPO.

Lane 3 - RainbowTM molecular weight markers.

An interesting feature of the denatured *B. subtilis* PPO was its ability to refold and regain activity on dilution (at least 50-fold) of the denaturing agents (6 M GuHCl, 8 M urea). During purification we observed apparent loss of flavin cofactor as the distinctive yellow colour associated with the flavins eluted in the void and some came off in the washes. Thus, this renaturation process also depended on apparent reassociation with the flavin cofactor which was present in the assay buffer and was essential for activity.

***M. xanthus* and Human PPOs: Transformation, Expression and Purification**

For purification of *M. xanthus* (figure 4.3) and human PPOs (figure 4.4), IPTG induction was not required. As mentioned in the “Methodology” no induction scheme

was followed as growth into stationary phase was sufficient for optimal induction. It would have been a fair assumption that the same purification procedure could be used for all three PPO's. However - all three purifications required modification (see Appendix 1) to obtain apparently pure PPO. Furthermore, the *M. xanthus* and human PPOs were easily purified on metal-affinity chromatography using non-denaturing conditions, whereas, in our hands, purification of *B. subtilis* PPO appeared unachievable using non-denaturing conditions.

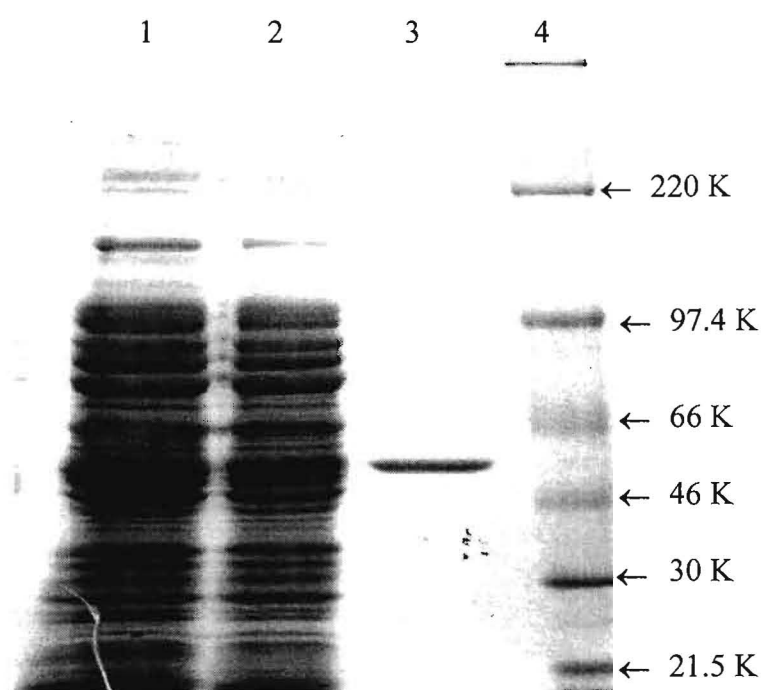


Figure 4.3: SDS-PAGE of the purification of *M. xanthus* PPO.

Lane 1 - supernatant from sonicated *E. coli* cells expressing *M. xanthus* PPO

Lane 2 - unbound proteins which fell through in the void volume

Lane 3 - purified *M. xanthus* PPO

Lane 4 – molecular weight markers.

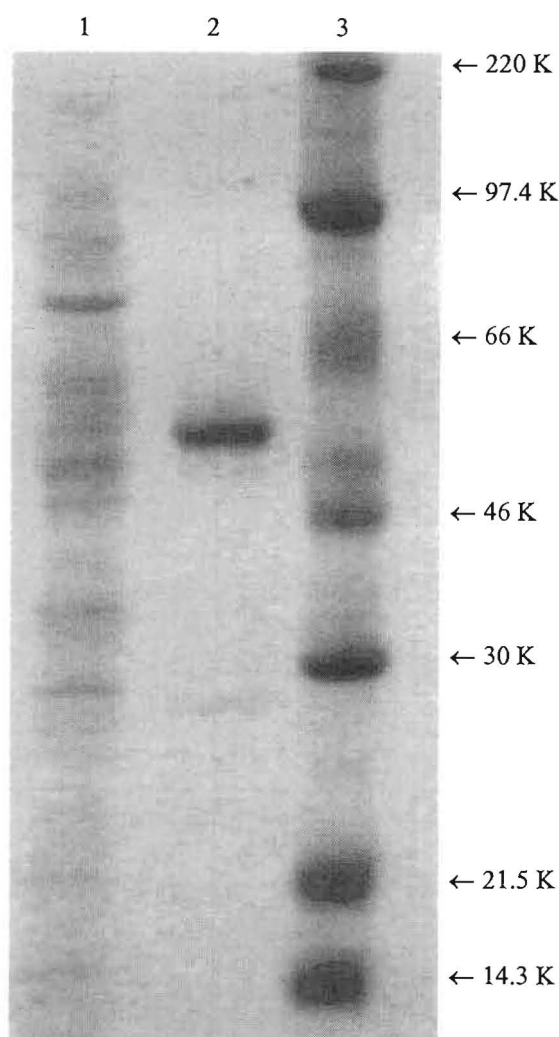


Figure 4.4: SDS-PAGE of the purification of human PPO.

Lane 1 - supernatant from sonicated *E. coli* cells expressing human PPO

Lane 2 - purified human PPO

Lane 3 - molecular weight markers.

In initial attempts to purify human PPO we utilised Ni-NTA agarose. The human PPO appeared to bind strongly to the Ni-NTA agarose column requiring a high imidazole (800 mM) concentration and 1 M NaCl in the elution buffer for elution. Even under these conditions a significant quantity appeared to remain bound to the column as judged by the presence of the (yellow) FAD. Thus we utilised Talon metal-affinity resin which gave a greater yield (5-6 mg) and better purity. Ni-based resins may bind non-6X His tagged proteins (QIA Expressionist, 1992). Although Talon binds 6X His proteins less tightly it has a significantly reduced affinity for non-6X His proteins.

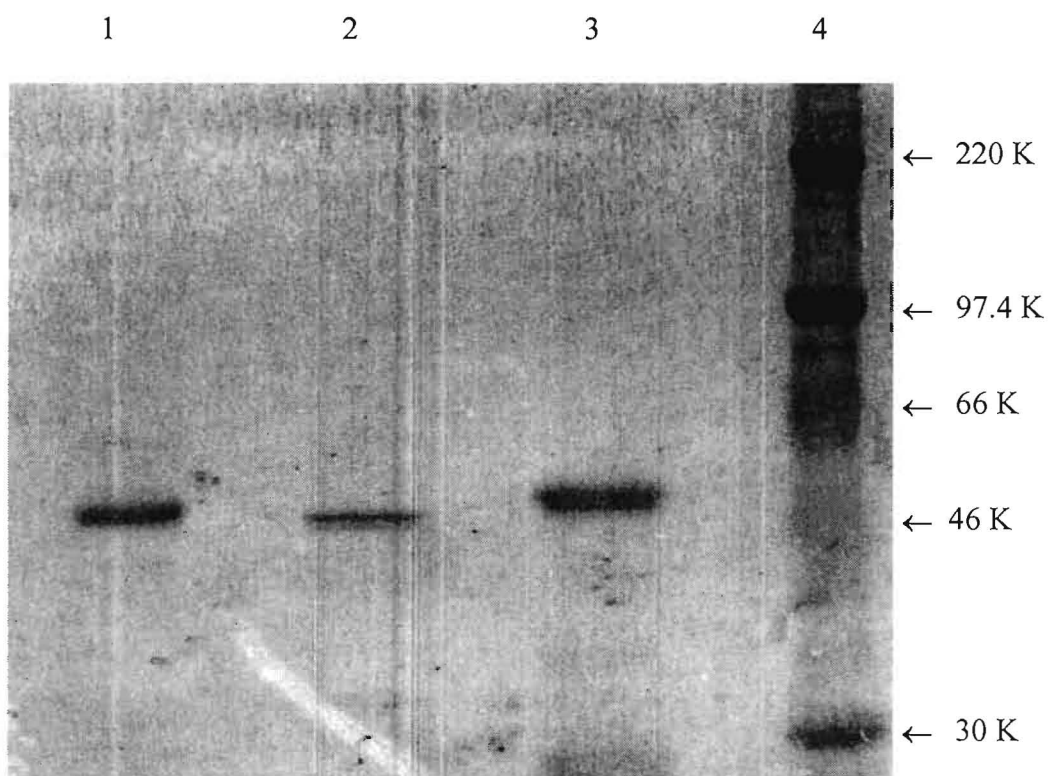


Figure 4.5: SDS-PAGE (7.5-17.5%) showing purification of the human, *M. xanthus* and *B. subtilis* PPOs in lanes 1, 2 and 3 respectively, using non-denaturing (native) conditions. The low molecular weight band at the bottom of lane 3 shows the partial purification of the *B. subtilis* PPO when using non-denaturing conditions. Lane 4 contains the molecular weight markers.

Concluding Remarks

A viable expression system was established for cloned *B. subtilis*, *M. xanthus* and human PPOs in our laboratory. The use of a pTrcHis expression system for the cloning of these proteins enabled purification of these proteins by a single-step procedure based on metal chelate affinity chromatography. This appears to be a great improvement to the purification of these proteins as previous methods involved the use of multiple chromatographic steps which contributed significantly to low protein yield at the end of a purification scheme (Klemm and Barton, 1987; Jacobs and Jacobs, 1987; Dailey and Karr, 1987; Siepker et al, 1987; Proulx and Dailey, 1992; Camadro et al, 1994).

These purifications allowed production of substantial amounts of apparently homogeneously pure PPOs with enzyme activity, which were suitable for further, selected, characterisation. The ability to produce and work with purified active PPOs facilitated the detailed characterisation of the *B. subtilis* PPO, and some aspects of the characteristics of PPO's from the other species purified could therefore be compared. This is described in the following chapters.

Chapter Five

Characterisation of Protoporphyrinogen Oxidases of *Bacillus subtilis*, *Myxococcus xanthus* and Human

Introduction

Early studies of the enzymatic oxidation of protoporphyrinogen-IX to protoporphyrin-IX utilised crude unpurified or partially purified PPO samples derived from membrane fractions of isolated mitochondria and chloroplasts, or the plasma membranes of prokaryotic cells (Jacobs and Jacobs, 1981; 1984a, b; Klemm and Barton, 1985; Camadro et al, 1985). The findings from these studies indicated that PPOs from different sources were rather different in terms of their molecular weights, subunit composition, associated cofactors, kinetic properties and terminal electron acceptors (Poulson and Polglase, 1975; Poulson, 1976; Jacobs and Jacobs, 1976, 1977a, b; Camadro et al, 1985; Klemm and Barton, 1987; Jacobs and Jacobs, 1987; Siepker et al, 1987; Dailey and Karr, 1987; Ferreira and Dailey, 1988; Proulx and Dailey, 1992). Importantly, these studies were crucial in demonstrating that the penultimate step of haem biosynthesis is catalysed by an intrinsic mitochondrial membrane protein (Deybach et al., 1985; Ferreira and Dailey, 1988). However, detailed kinetic analysis of these proteins was limited to a large extent by difficulties associated with their purification.

In the previous chapter we described the establishment of a viable expression system which facilitated the purification of the *B. subtilis*, *M. xanthus* and human recombinant PPOs to homogeneity using metal chelate affinity chromatography. This enabled us, to further characterise these proteins. The broad aim was thus to determine biophysical and kinetic properties of the *B. subtilis* PPO. For comparative purposes we used *M. xanthus* and human PPO and these were partially characterised as necessary.

Objectives

To determine:

- the M_r of recombinant *B. subtilis*, *M. xanthus* and human PPO and the subunit composition of *B. subtilis* PPO.
- the pH optima and kinetic rate constants of these PPOs with respect to the oxidation of protoporphyrinogen-IX to protoporphyrin-IX.
- the isoelectric point of *B. subtilis* PPO.

- the N-terminal amino acid composition of *B. subtilis* PPO.
- the substrate specificity of *B. subtilis* PPO.
- the flavin cofactors associated with the prokaryotic PPOs and the nature of their binding.
- the effects of various lipids on activity of the prokaryotic PPOs.

Methods and Materials

Determination of the relative M_r of the isolated *B. subtilis* PPO

The M_r of the *B. subtilis*, *M. xanthus*, and human PPOs were determined by polyacrylamide gel electrophoresis (SDS-PAGE) of the purified proteins and comparison with standard proteins of known molecular weight. Details are given in Appendices 2 and 3.

Briefly, gradient SDS-PAGE (7.5-17.5%) was performed under reducing conditions in 0.15% SDS on vertical slab gels. Approximately 4 μ g of PPO protein mixed in 1:1 ratio with sample loading buffer was loaded in each lane. The purified proteins and standard molecular weight markers were boiled for 5 min prior to loading, except for the denatured *B. subtilis* proteins which were incubated in a water bath at 37°C for 15 min before loading. The M_r 's of the purified PPO proteins were determined graphically by interpolation from plots of the molecular weights of the standard proteins against their relative migration distances through the gel.

Analysis of the subunit composition of the *B. subtilis* PPO

Gel filtration on a Sephadex G-200 column (1.6X 100 cm) in phosphate buffer (10 mM Na-phosphate, 100 mM NaCl, 0.2% *n*-octyl- β -D-glucopyranoside, pH 7.4) was done for mono-/multimeric analysis of non-denatured semi-purified *B. subtilis* PPO. SDS-PAGE was performed to determine the nature of the eluted protein. PPO flow through the column was compared to molecular weight markers from Pharmacia Biotech (Uppsala, Sweden).

Determination of the pH optima of *B. subtilis*, *M. xanthus* and human PPOs

The pH optima for the oxidation of protoporphyrinogen-IX by *B. subtilis*, *M. xanthus* and human PPOs were examined over the pH range 6 to 10, using the following buffer systems:

- 0.1 M potassium phosphate (pH range: 5 - 8.0)

- 0.1 M Tris (pH range: 7.2 - 9)
- 0.1 M carbonate (pH range: 9.0 - 10.7)

PPO activity was assayed in these buffer systems by measuring the constant velocity formation of protoporphyrin-IX from protoporphyrinogen-IX according to Meissner et al (1986), using the purified enzyme preparations. The enzyme activity was measured in the presence of the cofactor, FAD (5 μ M), to compensate for its potential loss during the purification of these proteins.

Determination of the rate constants, K_m and V_{max} , for the oxidation of protoporphyrinogen-IX by the different PPOs

The Michaelis-Menten constants (K_m) and maximal velocities (V_{max}), for the purified PPOs of *B. subtilis*, *M. xanthus* and human were determined by measuring the constant velocity formation of protoporphyrin-IX from protoporphyrinogen-IX in the substrate concentration range 0.1-30 μ M, during an incubation period of not more than 60 min. Approximately 4 μ g of enzyme were used in each 1 ml reaction mixture. The K_m and V_{max} values were determined from substrate-velocity plots created from sufficient representative points to give reasonable readings. Calculated values were determined with the computerised Gauss-Newton, iterative, non-linear curve fitting procedure.

B. subtilis PPO preparations from both denaturing and non-denaturing purification procedures were used in order to determine if the denaturation-renaturation process had any effects on the kinetics of the enzyme.

Determination of the isoelectric point (pI) of the purified *B. subtilis* PPO

B. subtilis PPO protein sample was desalted on a PD-10 column prepacked with Sephadex G-25 immediately before isoelectric focusing. Flat bed isoelectric focusing (pH 3.5-9.5) was performed on a 5% polyacrylamide gel. The isoelectric point was determined with the Pharmacia Broad pI Calibration Kit (pH range 3.0-10) using standard protein markers of known molecular weight as per manufacturer's instructions. Details are given in Appendix 6.

Amino acid composition of *B. subtilis* PPO

The amino acid composition of the N-terminal sequence of the purified *B. subtilis* PPO was determined by the Department of Biochemistry, University of Cape Town. Approximately 100 μ g of protein was subjected to 6N HCl hydrolysis, in vacuo, for 24 h. Amino acids were separated using the Waters HPLC amino acid analysis protocol employing a CX pak strong cation exchange column, and detected by O-phthalaldehyde (OPA) derivatisation. When proline was determined, the OPA-hypochloride detection system was used.

Determination of substrate specificity of the recombinant *B. subtilis* PPO

The substrate specificity of the *B. subtilis* PPO was determined by examining the oxidation of protoporphyrinogen-IX, coproporphyrinogen-III, mesoporphyrinogen-IX, and uroporphyrinogen-III by this enzyme. For determination of porphyrin concentrations, HCl was used as a solvent at concentrations suitable for dissolving the various porphyrin compounds (table 5.1). The extinction coefficients (ϵ mM) of the different porphyrins were used to calculate their concentrations in these solutions after measurement of their maximum absorbances.

Table 5.1: Porphyrin compounds tested with the *B. subtilis* PPO.

[Porphyrin] μ M = O.D. $_{\lambda_{\max}}$ / ϵ mM X Dilution factor

Porphyrin Substrate	[Solvent]	λ_{\max} (nm)	ϵ mM
Protoporphyrinogen-IX	2.7N HCl	405	262
Mesoporphyrinogen-IX	0.1N HCl	399	445
Coproporphyrinogen-III	0.1N HCl	399.5	489
Uroporphyrinogen-III	0.5N HCl	405.5	541

The assays for the different porphyrin substrates were performed at pH 8.7 in assay buffer. Approximately 4 μ g of purified PPO were added in each reaction. Reduction of the porphyrins to corresponding porphyrinogens with 4% sodium amalgam was done immediately prior to the beginning of the assay. Kinetic constants for each of the porphyrin compounds were determined as outlined above. The data for each of the substrates tested represents the mean of at least three independent sets of results obtained from assays carried out on different enzyme preparations on different days.

Analysis of the flavin cofactors associated with *B. subtilis* and *M. xanthus* PPOs

Putative flavins were extracted from the relevant PPO preparations and analysed by spectrophotometry. Details are given in Appendix 7. Briefly, a TCA protein precipitate was removed by centrifugation and the flavin-containing acidic supernatant was immediately adjusted to pH 7.4 with 3 M Tris and kept at 4°C in the dark to avoid hydrolysis and photodegradation of flavin. An emission fluorescence spectrum for this supernatant was then determined. The spectra were compared to similar recordings performed for FAD and FMN standards.

Effects of various lipids on activity of *B. subtilis* and *M. xanthus* PPOs

The activities of *B. subtilis*, and *M. xanthus* PPOs were assayed in the presence of palmitic (Sigma Products) and oleic acid (UniLAB) using sufficient representative points (9-15) over a range of lipid concentrations from 0-280 μ M. Ethanol was used to dissolve the lipids giving a final solvent concentration of 2.5% in the reaction mixture for both samples and blanks. The experiments were repeated on three separate enzyme preparations and the results averaged.

Results and Discussion

Molecular weight determinations and subunit composition of purified *B. subtilis* PPO

SDS-PAGE of human, *B. subtilis*, and *M. xanthus* PPOs yielded apparent M_r 's of ~53 000, 56 000 and 52 400 Da, respectively (Figure 5.1 a, b, c). This was in agreement with other cloned, purified and characterised PPOs with molecular weights reported in the 52 000 - 56 000 Da range, such as the mouse (Dailey et al, 1995; Taketani et al, 1995a); *A. thaliana* (Narita et al, 1996); yeast (*S. cerevisiae*) (Camadro et al, 1994); tobacco plant (Lermontova et al, 1997); spinach (Matringe et al, 1992a) and potato plant (Johston et al, 1998).

The slightly higher M_r 's of our recombinant proteins than those predicted from their derived amino acid sequences may be attributed to the presence of the 6His tag at their N-termini. It has been reported that proteins with the 6His tag may run slower, during gel electrophoresis, than the equivalent untagged proteins (The QIA Expressionist, 1992).

Gel filtration chromatography of the nondenatured partially purified *B. subtilis* PPO performed on a Sephadex G-200 column yielded a monomer of ~ 54 000 Da (figure 5.2) confirming previous findings based on HPLC filtration of the recombinant protein expressed from a pBTac1 plasmid which also showed that the active *B. subtilis* PPO existed as a 53 000 Da monomer (Dailey et al, 1994a). *B. subtilis* PPO appears to be different to both the human and *M. xanthus* recombinant PPOs which were previously shown to be homodimers of 100 000 Da comprised of subunits of approximately 50 000 Da (Dailey and Dailey, 1996a, b).

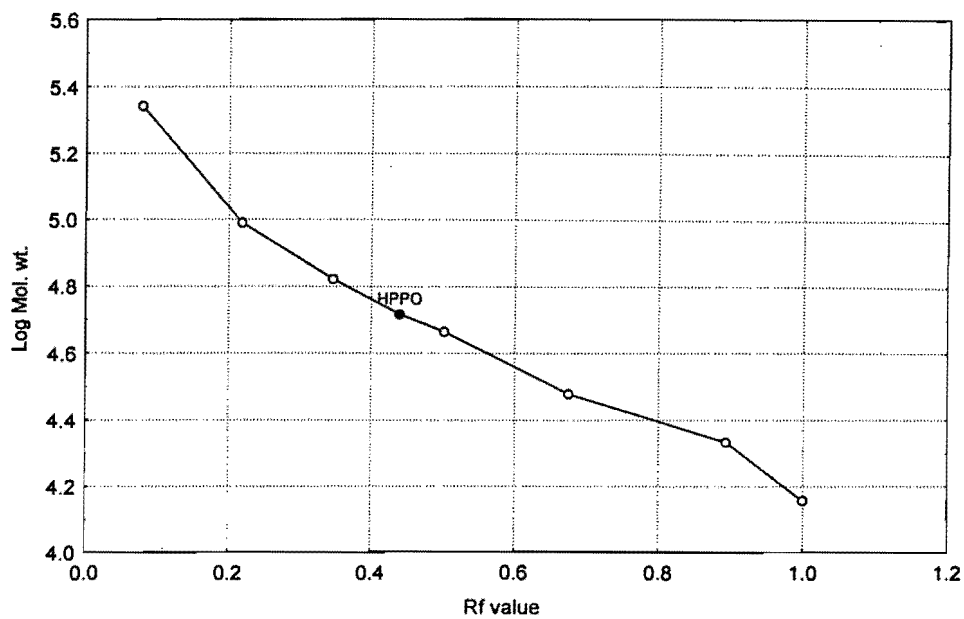


Figure 5.1a: Calibration curve for determination of molecular weight of the purified human PPO. This yielded a M_r of approximately 53 000 Da.

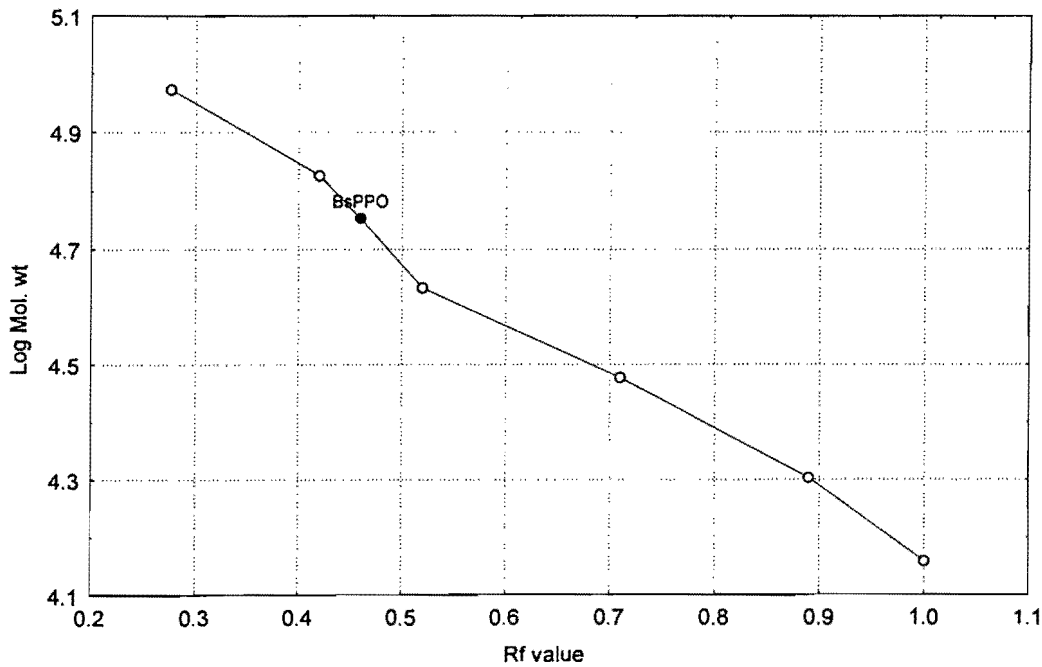


Figure 5.1b: Calibration curve for determination of molecular weight of the purified *B. subtilis* PPO. This yielded a M_r of approximately 56 000 Da.

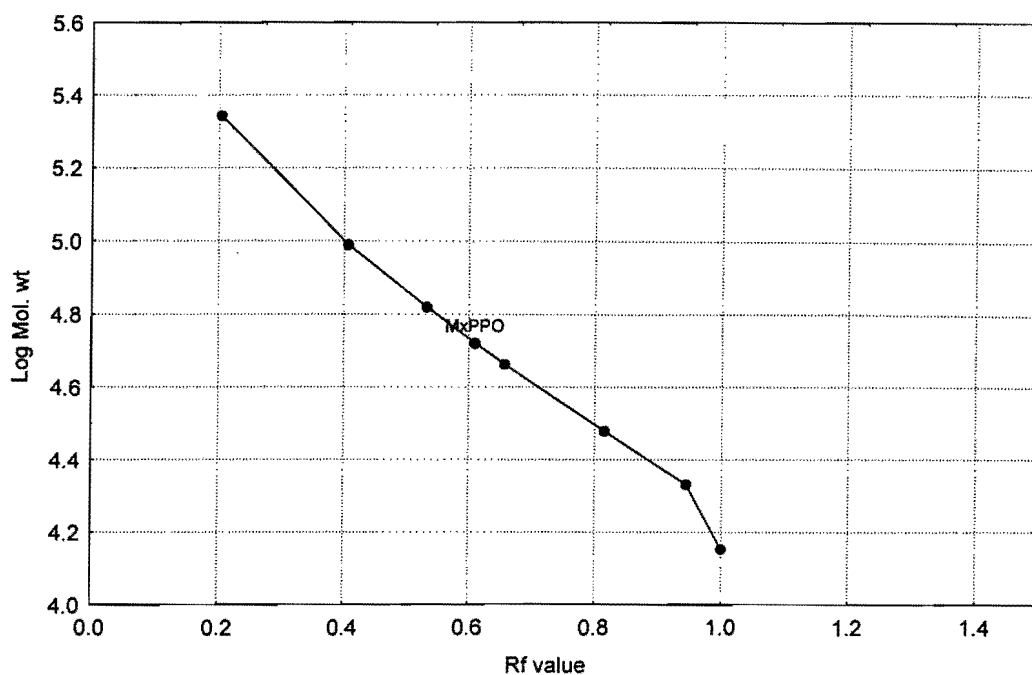


Figure 5.1c: Calibration curve for determination of molecular weight of the purified *M. xanthus* PPO. A M_r of approximately 52 400 Da was obtained.

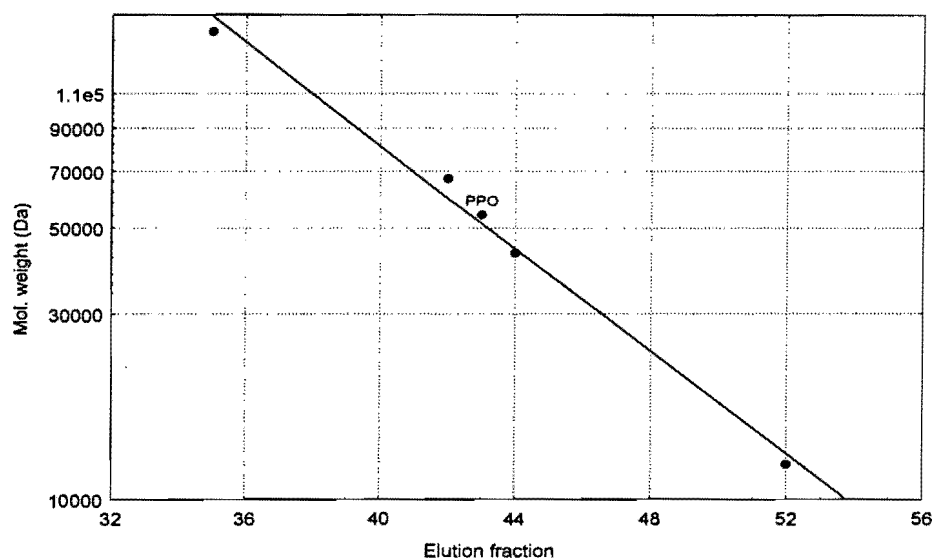


Figure 5.2: Determination of molecular weight of native *B. subtilis* PPO using Sephadex G200 gel filtration. This protein eluted as a 54 000 Da monomer. The molecular weight markers used were Aldolase (158 000 Da), Bovine serum albumin (67 000 Da), Ovalbumin (43 000 Da) and Cytochrome c (12 300 Da).

pH optima of *B.subtilis*, *M. xanthus* and human PPOs

The pH optima for the oxidation of protoporphyrinogen-IX by the PPOs of *B. subtilis*, *M. xanthus* and human were 8.7, 8.1 and 8.1 respectively (Figures 5.3, 5.4, and 5.5).

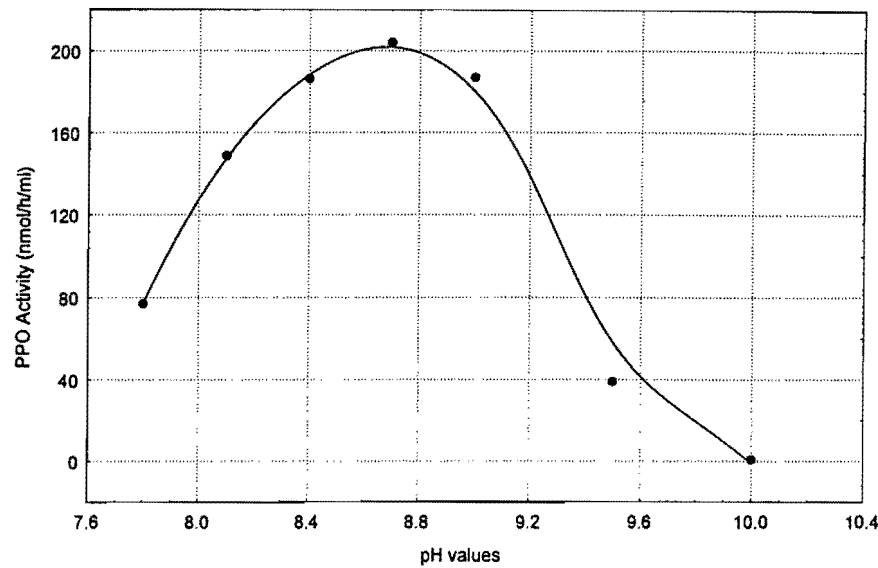


Figure 5.3: Effect of pH on activity of *B. subtilis* PPO. Optimum oxidation of protoporphyrinogen-IX by the *B. subtilis* PPO occurred at pH 8.7 ($n = 3$).

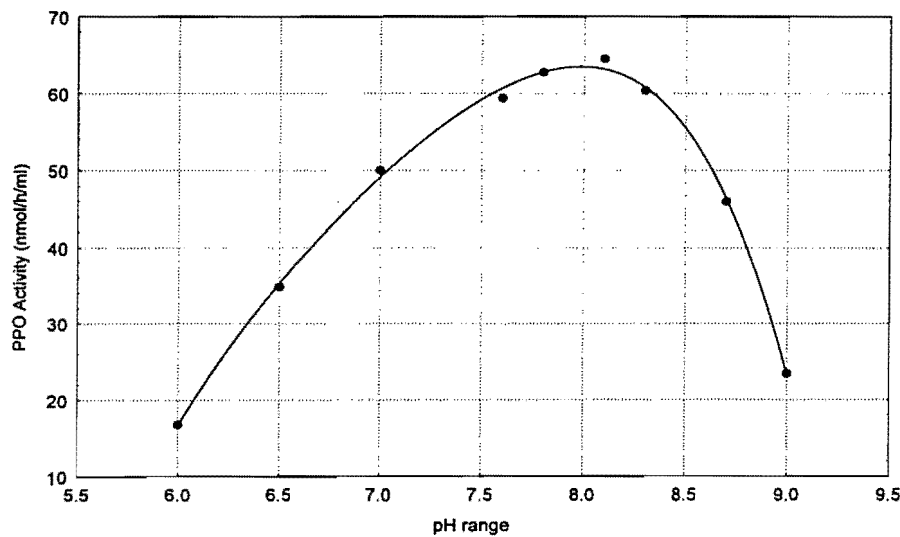


Figure 5.4: Effect of pH on activity of *M. xanthus* PPO. Optimum oxidation of protoporphyrinogen-IX by the *M. xanthus* PPO occurred at pH 8.1 ($n = 3$).

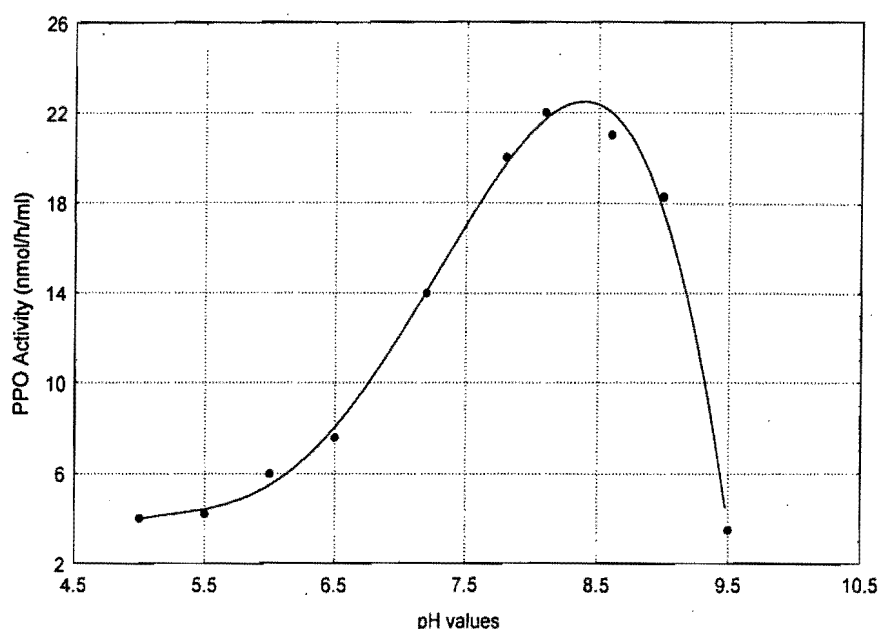


Figure 5.5: Effect of pH on activity of Human PPO. Optimum oxidation of protoporphyrinogen-IX by the human PPO occurred at pH 8.1 ($n = 3$).

The pH optima of other characterised PPOs such as the rat (8.7) (Poulson, 1976); mouse (8.0) (Ferreira and Dailey, 1988), and bovine (8.7) (Siepker et al, 1987) were also within this basic range. This is in contrast to the multi-subunit, acidic PPO of the anaerobic bacterium, *D. gigas* which appears to tolerate changes in pH (Klemm and Barton, 1987). Optimum activity of barley etioplastic PPO was reported to occur at pH 7.3 and that of the spinach enzyme at pH 7.0 (Jacobs and Jacobs, 1984a). Similarly, the yeast PPO oxidises protoporphyrinogen-IX optimally at pH 7.2 to 7.4 (Poulson and Polglase, 1975; Camadro et al, 1994). A further report on barley PPO suggests an acidic pH optimum of 6.0 (Jacobs and Jacobs, 1987) although this may not reflect its operational pH *in vivo* as there would be significant auto-oxidation at this pH.

Rate constants for the oxidation of protoporphyrinogen-IX by the different PPOs

The oxidation of protoporphyrinogen-IX to protoporphyrin-IX by the purified *B. subtilis* PPO obeyed Michaelis-Menten kinetics as indicated by the typical saturation of enzyme by high concentrations of substrate (figure 5.6a) which yields a straight line when the double-reciprocal is plotted (Lineweaver-Burke plot) (figure 5.6b)). Similar curves were obtained for both the purified *M. xanthus* and human PPOs with respect to oxidation of protoporphyrinogen-IX.

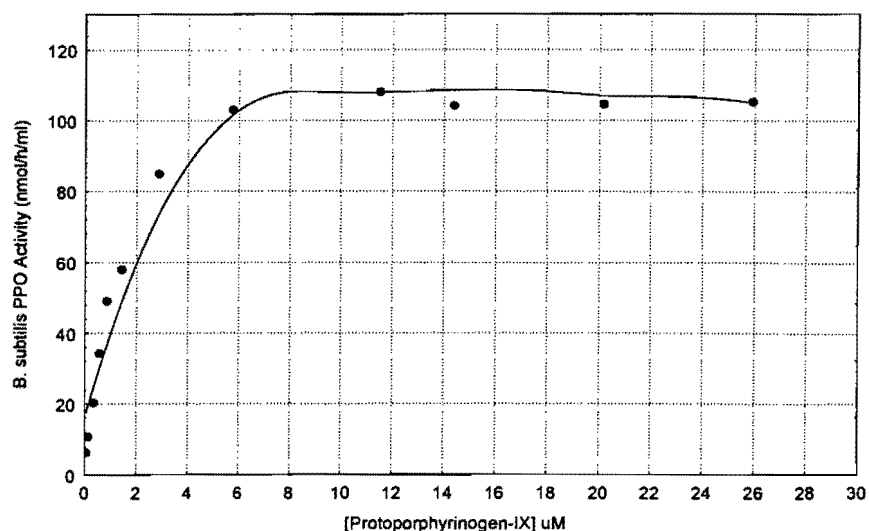


Figure 5.6 (a): Substrate-velocity plot for the enzymatic oxidation of protoporphyrinogen-IX by *B. subtilis* PPO.

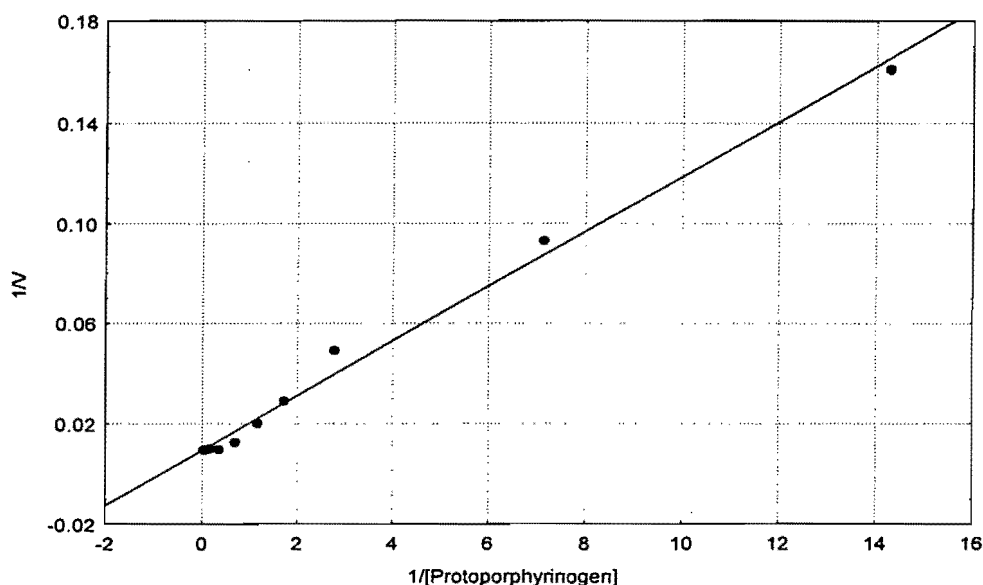


Figure 5.6 (b): Lineweaver-Burke plot of the oxidation of protoporphyrinogen-IX by the *B. subtilis* PPO (data from figure 5.6a).

V_{\max} and K_m values for *B. subtilis* (denatured and partially pure non-denatured), *M. xanthus* and human PPO are given in Table 5.2. The K_m for protoporphyrinogen-IX oxidation by *B. subtilis* PPO was similar to that reported by Hansson and Hederstedt (1994) for *B. subtilis* HemY protein.

Table 5.2. Michaelis-Menten constants (K_m) and maximal velocities (V_{max}) for the oxidation of protoporphyrinogen-IX by the different PPO species. In all cases $n=4$.

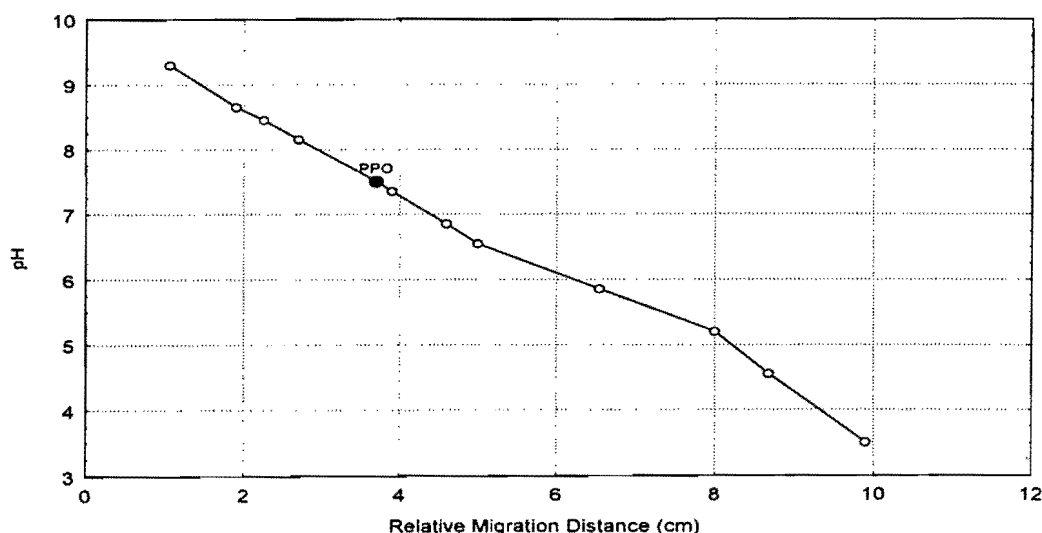
PPO	K_m (μM)	V_{max} (nmol/min/mg)
<i>B. subtilis</i> (renatured)	1.00 ± 0.15	3.70 ± 0.12
<i>B. subtilis</i> (nondenatured)	1.14 ± 0.15	3.87 ± 0.21
<i>M. xanthus</i>	0.40 ± 0.03	8.80 ± 0.20
Human	0.67 ± 0.29	734.00 ± 39

The denaturing conditions used in the purification of the *B. subtilis* PPO did not affect the ability of the enzyme to oxidise protoporphyrinogen-IX as indicated by the similar K_m and approximate V_{max} values for the nondenatured (native) and renatured proteins. It would appear therefore, that the renatured PPO refolds correctly.

While it was of concern that kinetic studies on the *B. subtilis* PPO were to be carried out on a protein purified under denaturing conditions, the K_m 's and V_{max} 's for the oxidation of protoporphyrinogen-IX by PPO under these conditions were in good agreement with those of the non-denatured semipurified enzyme.

Isoelectric point (pI) of the purified *B. subtilis* PPO

Analytical isoelectric focusing yielded an experimental pI of 7.5 (figure 5.7). This was in reasonable agreement with that of 8.3 predicted from the amino acid sequence derived from the *hemY* gene (Dailey et al, 1994a), and with those of 8.0, 8.5 and 8.82 calculated for the purified human (Dailey and Dailey, 1996b), yeast (Camadro et al, 1994) and mouse (Dailey and Karr, 1987) PPOs, respectively. Thus, it appears that most PPOs have similar pI values in the neutral to weakly basic range, pH 7.5-8.8. However, the *D. gigas* enzyme is an exception in that it has a reported pI of 5.71 (Klemm and Barton 1987).



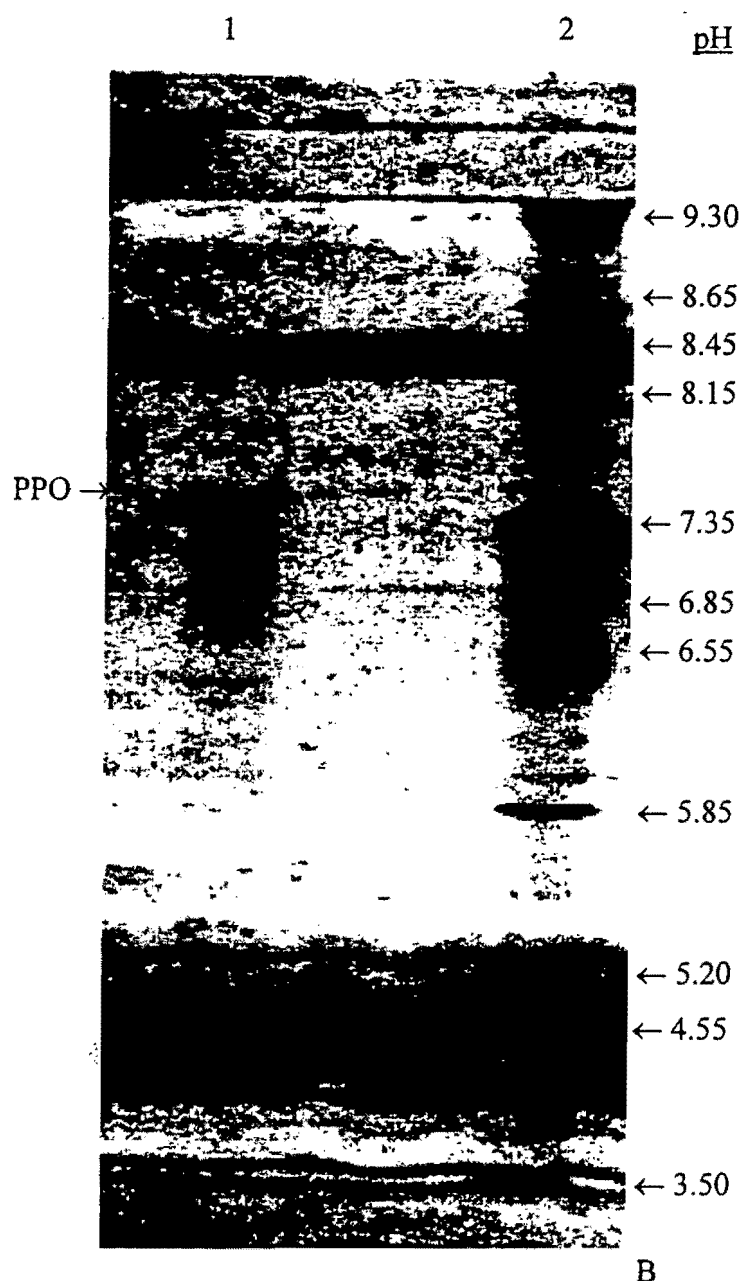


Figure 5.7. Flatbed isoelectric focusing of the *B. subtilis* PPO was performed on a 5% polyacrylamide gel (B), showing in lane 1 – *B. subtilis* PPO, and lane 2 – the Pharmacia Broad pI standard protein markers. The experimental isoelectric point of the purified PPO was calculated from the plot of pH vs relative migration distance (A).

The amino acid composition of *B. subtilis* PPO

The experimentally determined amino acid composition of the purified *B. subtilis* PPO (Table 5.3) was in reasonable agreement with the published amino acid sequence derived from the *hemY* (PPO) gene sequence (Hansson and Hederstedt, 1992; Dailey et al, 1994a). This allowed us to confirm identity of the protein.

Table 5.3: Chromatogram profile of the amino acid analysis of pure *B. subtilis* PPO. N.d. – not determined. Predicted amino acid composition from *HemY* gene sequence (Hansson and Hederstedt, 1992).

Amino Acid	Mol %	Predicted Mol %
Asp	6.37	4.70
Glu	8.54	10.43
Ser	6.78	7.45
Thr	6.35	6.38
Gly	10.69	8.94
Ala	7.18	7.45
Arg	3.38	3.19
Pro	5.40	4.47
Val	7.35	7.02
Met	3.57	2.98
Ile	5.88	5.74
Leu	11.29	10.00
Phe	3.58	3.19
Cys	N.d.	0.85
Lys	8.64	8.30
His	3.14	2.77
Tyr	1.86	2.55

Substrate specificity of the purified *B. subtilis* PPO

In addition to the natural PPO substrate (protoporphyrinogen-IX) both coproporphyrinogen-III, and the non-physiologic mesoporphyrinogen-IX were utilized as substrates by the *B. subtilis* PPO. Uroporphyrinogen-III was not oxidised by this PPO. The oxidation of all the substrates by this enzyme followed classical Michaelis-Menten kinetics. The K_m , V_{max} , k_{cat} and k_{cat}/K_m values for the oxidation of the various substrates are summarised in table 5.4. k_{cat} , sometimes referred to as the turnover number, represents the maximum number of substrate molecules converted to product per active site of enzyme molecule in a unit time.

Table 5.4. Michaelis-Menten (K_m), maximum velocity (V_{max}) and catalytic constants (k_{cat} , k_{cat}/K_m) for the various substrates oxidised by the *B. subtilis* PPO.

Substrate	K_m (μ M)	V_{max} (nmol/min/mg)	k_{cat} (min^{-1})	k_{cat}/K_m
Protoporphyrinogen	1.00 ± 0.15	3.70 ± 0.12	0.19 ± 0.030	0.19
Coproporphyrinogen	5.29 ± 0.36	0.98 ± 0.09	0.05 ± 0.005	0.01
Mesoporphyrinogen	4.92 ± 0.75	45.94 ± 6.00	2.69 ± 0.600	0.55

The efficiency for the oxidation of the different substrates by the *B. subtilis* PPO was in the order, $K_{cat}(\text{mesoporphyrinogen}) > K_{cat}(\text{protoporphyrinogen}) > K_{cat}(\text{coproporphyrinogen})$. It is of interest to compare the apparent specificity constants K_{cat}/K_m as this relates the reaction rate to the concentrations of free rather than total enzyme. This preference of the *B. subtilis* PPO for mesoporphyrinogen is in contrast to mammalian PPO. Human liver mitochondrial PPO has preference for protoporphyrinogen (apparent K_m 0.16 μ M, V_{max} 8.5 nmol/h/mg) over mesoporphyrinogen which has two different apparent K_m s of 0.5 μ M (V_{max} 2.4 nmol/h/mg) and 4 μ M (V_{max} 5.7 nmol/h/mg) (Camadro et al, 1985). Similarly mouse liver PPO has tenfold higher activity with protoporphyrinogen compared to mesoporphyrinogen (Dailey and Karr, 1987). Coproporphyrinogen-III, the least preferred of the *B. subtilis* substrates, is not utilised by the purified human enzyme (Dailey and Dailey, 1996b).

This is in contrast to the findings by Hansson and Hederstedt (1994) who reported preference by the *B. subtilis* *hemY* protein for coproporphyrinogen-III over protoporphyrinogen-IX. The $K_{m(\text{app})}$ s for protoporphyrinogen and coproporphyrinogen were 1.00 μ M and 5.3 μ M respectively in contrast to 0.95 μ M and 0.56 μ M reported by these workers. In addition we obtained a higher V_{max} for protoporphyrinogen than for coproporphyrinogen in contrast to these workers ($V_{max}(\text{coproporphyrinogen})$ 7.0 nmol/min/mg; $V_{max}(\text{protoporphyrinogen})$ 0.85 nmol/min/mg). Some of the differences in calculated kinetic parameters may be attributed to the problematic nature of substrate preparation and quantitation, differences in the assay medium (in particular our pH of 7.2 vs. 8.7 of Hansson and Hederstedt (1994)) as well as rates of endogenous (non-enzymatic) porphyrinogen oxidation. However, it is surprising that there are qualitative differences in the comparative kinetic values reported for copro- versus protoporphyrinogen.

This enzyme was different to both human (Dailey and Dailey, 1996b) and *M. xanthus* (Dailey and Dailey, 1996a) PPOs which could not oxidise coproporphyrinogen-III. However, the fact that the *B. subtilis* PPO could not oxidise uroporphyrinogen-III showed that it is not a general porphyrinogen oxidase.

The reported inability to oxidise coproporphyrinogen-III by the *M. xanthus* PPO (Dailey and Dailey, 1996a and M. Maneli- personal communication, our laboratory) showed that the broad substrate specificity was not a general prokaryotic property of these enzymes.

Both protoporphyrinogen-IX and mesoporphyrinogen-IX are dicarboxylic porphyrins, whilst coproporphyrinogen-III is a tetracarboxylic porphyrin. Mesoporphyrinogen-IX differs from protoporphyrinogen-IX by the substitution of the vinyl groups at positions 2 and 4 of the tetrapyrrole macrocycle with ethyl groups. This structural resemblance between these compounds explains their recognition and high affinity for the *B. subtilis* PPO. Uroporphyrinogen-III on the other hand is an octacarboxylic porphyrin, and sterically larger than the other porphyrinogens and this may limit its interaction with the active site of this enzyme.

The preference by the *B. subtilis* PPO for its natural substrate, protoporphyrinogen-IX over coproporphyrinogen-III suggests that this organism probably contains a non-PPO enzyme which catalyses the conversion of coproporphyrinogen-III to protoporphyrinogen-IX for the normal biosynthesis of haem. In several organisms this reaction was shown to be catalysed by two enzymatic systems that is, an oxygen requiring coproporphyrinogen oxidase (*hemF*) and an oxygen-independent coproporphyrinogen oxidase (*hemN*) (Tait, 1969, 1972; Seehra et al., 1982). Following recent studies which showed that *B. subtilis* could exist anaerobically via nitrate ammonification (Hoffmann et al, 1995), in contrast to previous beliefs that it was a strict aerobe, an open reading frame which had amino acid homology to the oxygen-independent coproporphyrinogen oxidase (*hemN*) was discovered in this organism (Homuth et al, 1995). This protein was subsequently shown to be involved in oxygen-independent decarboxylation of coproporphyrinogen-III to protoporphyrinogen-IX (Hippler et al, 1997). The oxygen-dependent coproporphyrinogen oxidase (*hemF*) of *B. subtilis* has not yet been discovered.

Determination of the flavin cofactors associated with the prokaryotic PPOs of *B. subtilis* and *M. xanthus*.

A yellow substance, presumed to be flavin cofactors, associated with the *B. subtilis* and *M. xanthus* PPOs was extracted from the purified proteins with cold 10% TCA. The fluorescence emission spectrum (Excitation wavelength = 465 nm) yielded three peaks in the region of 370, 435 and 520 nm (figure 5.8); and this was indicative of an oxidized flavin cofactor (Koziol, 1971, Ghisla, 1980).

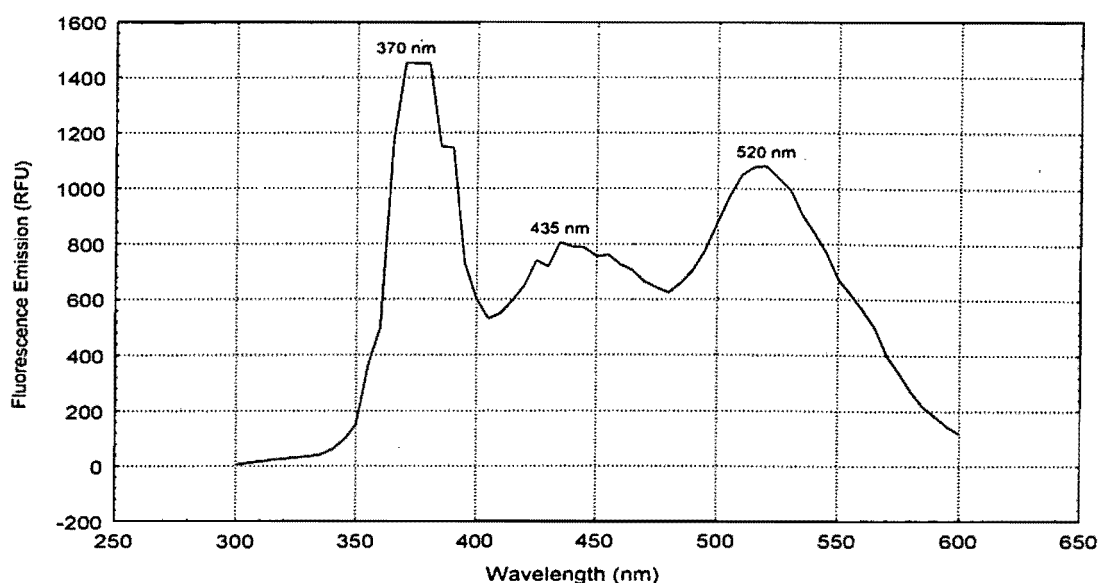


Figure 5.8.: Fluorescence emission spectrum of the flavin cofactor released from the *B. subtilis* PPO during purification, measured at an excitation wavelength of 465 nm. RFU = Relative Fluorescence Units.

Moreover, loss of the yellow colour on reduction of this compound with dithiothreitol (DTT) and restoration of PPO activity after purification under denaturing conditions and addition of exogenous FAD was further indicative of a flavin compound (figure 5.9). A concentration-dependent increase in oxidation was observed up to a final assay FAD concentration of 4 μM , and thereafter no further increase occurred suggesting that the enzyme was saturated with cofactor.

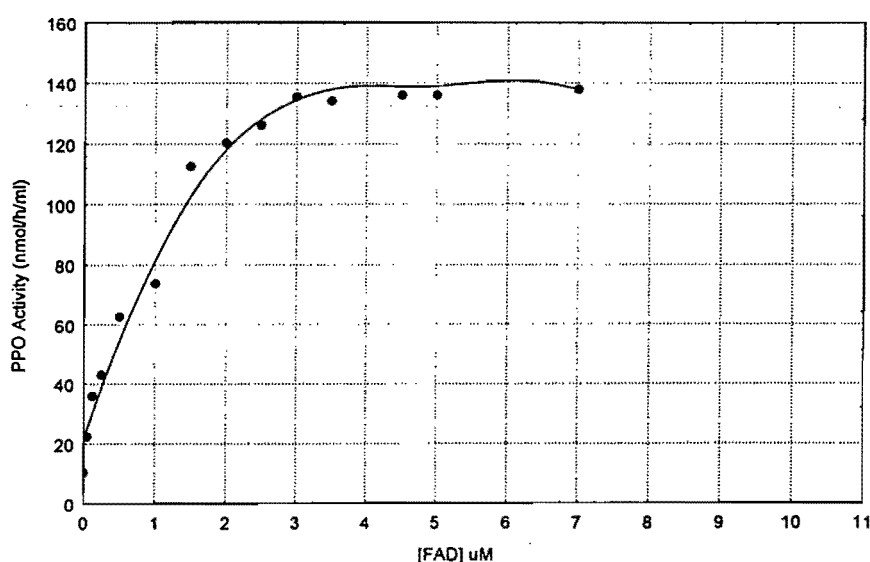


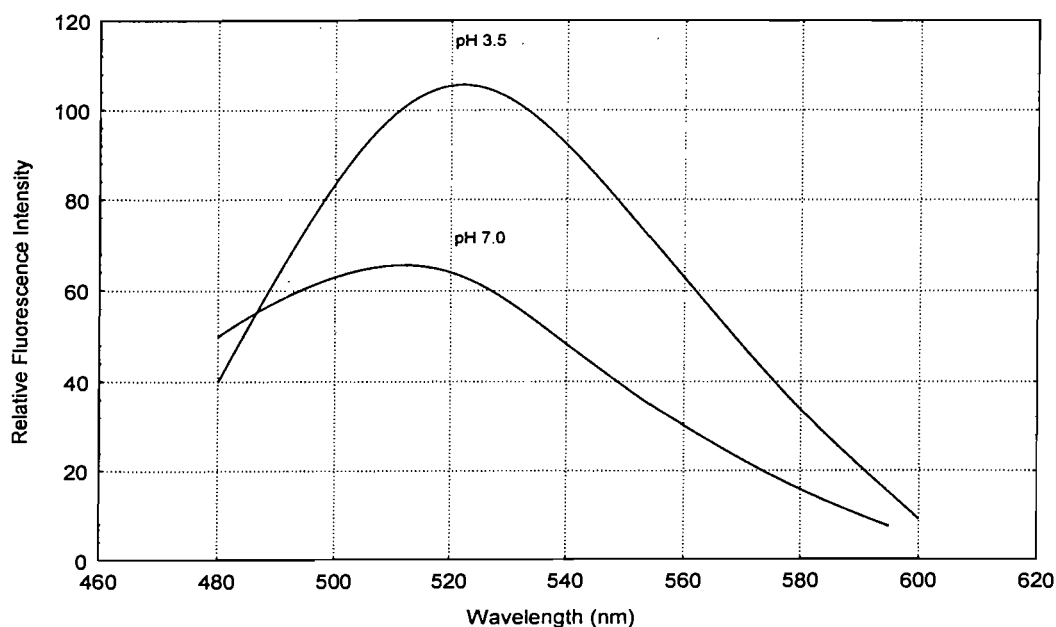
Figure 5.9. The effects of FAD on the oxidation of protoporphyrinogen-IX by the renatured *B. subtilis* PPO (4 μg). Reactivation of the enzyme occurred on addition of commercial FAD to the assay reaction.

The fluorescence emission spectra of the cofactors extracted from the purified *B. subtilis* (figure 5.10 (A)) and *M. xanthus* (figure 5.10 (B)) PPOs, measured in acidic

(pH 3.5) and neutral (pH 7.0) solutions, showed a characteristic FAD pH-dependent spectral shift, confirming previous findings that this was the cofactor associated with both the *B. subtilis* (Dailey et al 1994a) and *M. xanthus* (Dailey and Dailey, 1996a) PPOs.

The relative ease of extraction of the flavin cofactors from their respective PPOs (10% TCA) suggests that they are noncovalently associated with the protein which is similar to a report for human PPO (Dailey and Dailey, 1996b). In contrast, the FAD and FMN cofactors reportedly associated with the bovine (Siepker et al., 1987) and mouse (Proulx and Dailey, 1992) enzymes, respectively, were suggested to be covalently bound to these proteins, as they needed trypsin digestion or hot mineral acids for extraction.

Interestingly, detailed fluorometric analysis of the reportedly purified plant (barley) PPO could not reveal presence of chromophoric compounds (Jacobs and Jacobs, 1987; Jacobs et al., 1989). However, evidence at the primary structural level has revealed presence of the dinucleotide binding ($\beta\alpha\beta$) motif in *A. thaliana* (Narita et al., 1996) and tobacco plant (Lermontova et al., 1997) PPOs, suggesting that these are also flavoproteins. The PPO from *S. cerevisiae* was also shown to be an FAD-containing flavoprotein (Camadro et al., 1994).



A

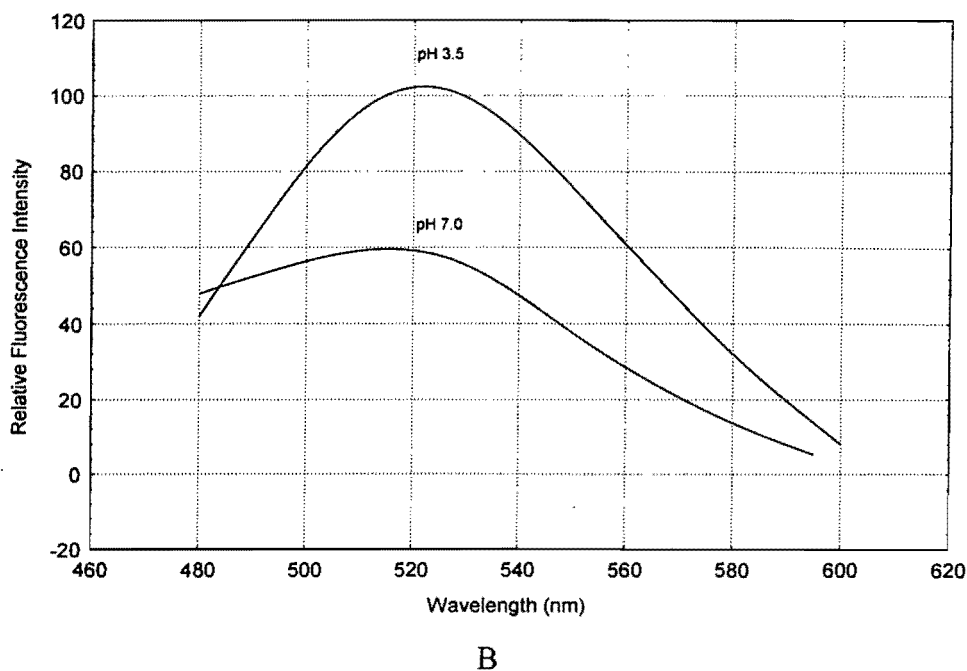


Figure 5.10: The fluorescence emission spectra of the flavin cofactors extracted from the *B. subtilis* (A) and *M. xanthus* (B) PPOs, measured from 600 to 480 nm using an excitation wavelength of 450 nm, revealed a pH-dependent spectral shift typical of FAD.

In the light of the above discussion we would suggest that PPO activity is critically dependent on the presence of a flavin cofactor to facilitate oxidation of protoporphyrinogen-IX for haem biosynthesis.

Effects of lipids on PPO activity

The addition of oleic or palmitic acid generally resulted in an inhibition of *B. subtilis* PPO activity although there appeared to be slight stimulation activity at low concentrations (figures 5.11 and 5.12). The presence of the fatty acids caused slight increase in the rate of auto-oxidation of protoporphyrinogen-IX but this was controlled for in the assay. The results represents means of data from three independent sets of experiments.

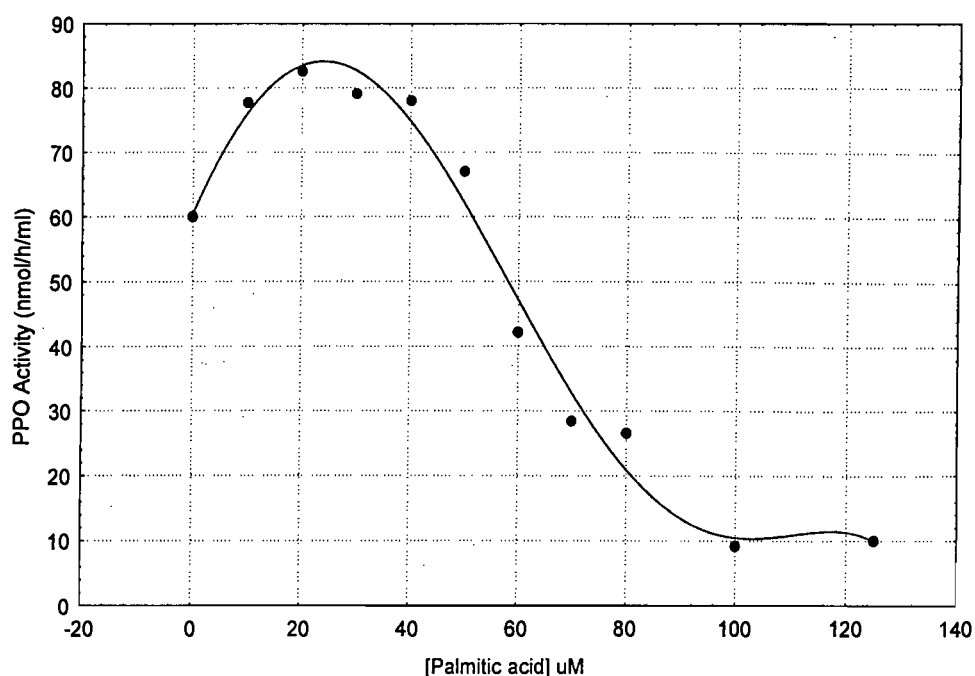


Figure 5.11. Palmitic acid concentrations up to approximately 20 μM had a slight stimulatory effect on the *B. subtilis* PPO activity. Concentrations above this had an inhibitory effect on the enzyme activity.

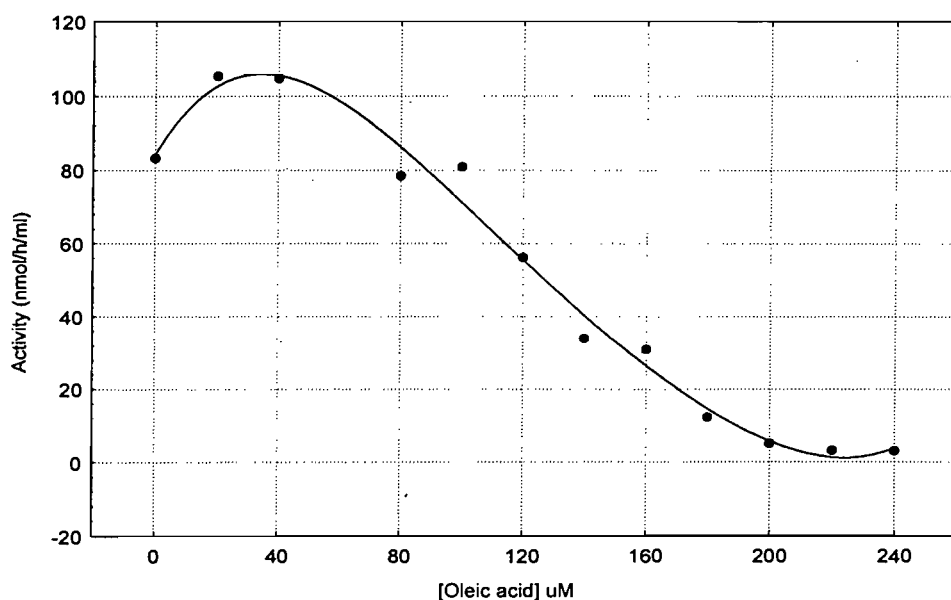


Figure 5.12. Oleic acid concentrations up to approximately 20 μM had a slight stimulatory effect on the *B. subtilis* PPO activity. Concentrations above this had an inhibitory effect on the enzyme activity.

In contrast to the *B. subtilis* PPO, oleic and palmitic acid had stimulatory effects on the *M. xanthus* PPO activity up to approximately 100 μM and 80 μM , respectively (figure 5.13 and 5.14).

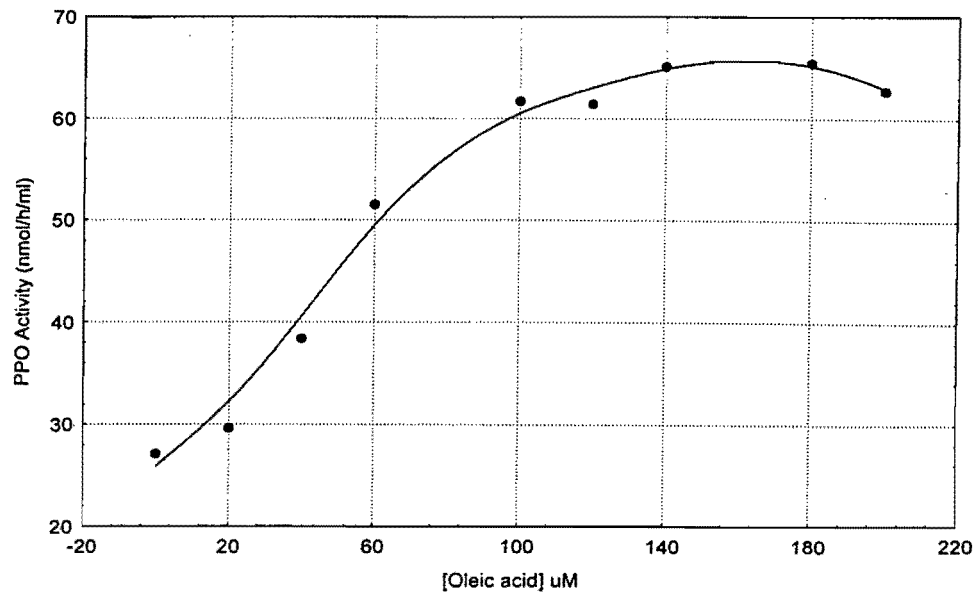


Figure 5.13. Oleic acid stimulated *M. xanthus* PPO activity up to approximately 100 μ M.

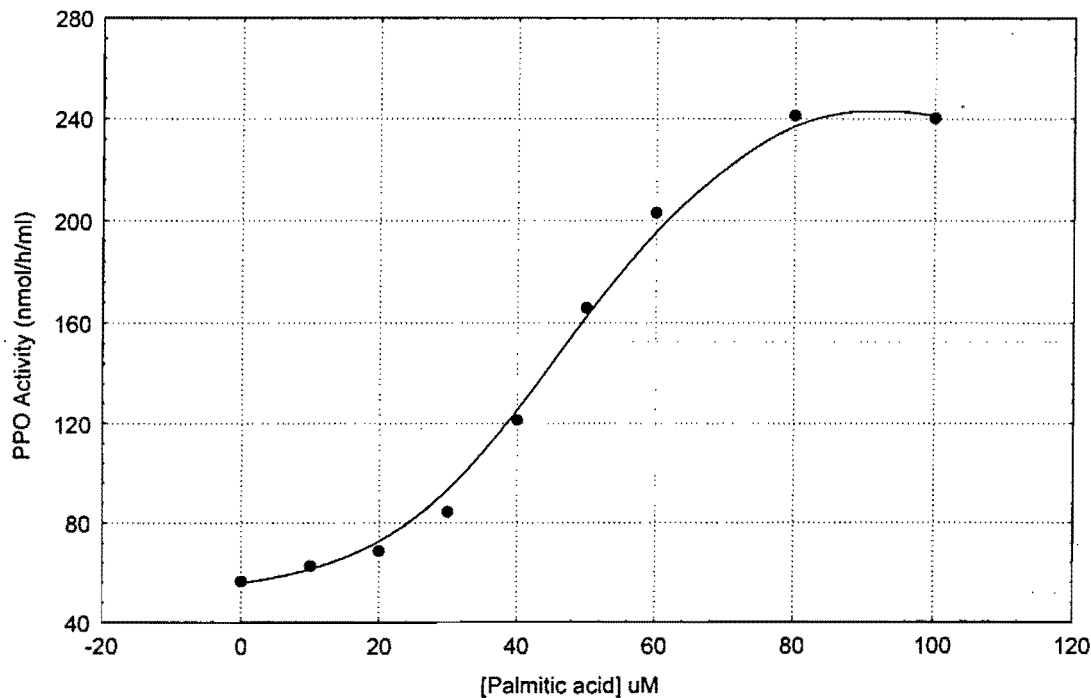


Figure 5.14. Effect of palmitic acid on *M. xanthus* PPO activity was also stimulatory up to approximately 80 μ M.

The stimulation of the *M. xanthus* PPO by enhancement of the hydrophobic environment is in keeping with the concept that PPO is primarily membrane bound. The lack of stimulation of the *B. subtilis* PPO by the addition of fatty acids lends support to the findings that *B. subtilis* PPO exists partially in the soluble cytoplasmic fraction, unlike all other reported PPO's which are membrane bound.

Concluding Remarks

This data illustrates differences and similarities in the characteristics of *B. subtilis*, *M. xanthus* and human PPOs. A striking difference is the ability of the *B. subtilis* enzyme to utilise not only the common substrate for all PPOs, protoporphyrinogen, but also coproporphyrinogen and mesoporphyrinogen. Furthermore, the behaviour of *B. subtilis* PPO in the presence of lipids is unlike that of another prokaryote studied, *M. xanthus*.

These differences led us to further investigate the kinetic behaviour of *B. subtilis* PPO by way of inhibitor studies. These are described in the following chapter.

Chapter Six

Inhibition Studies on *Bacillus subtilis*, *Myxococcus xanthus* and Human Protoporphyrinogen Oxidases

Introduction

This work provides detailed kinetic data on the inhibition of human, *B. subtilis* and *M. xanthus* PPOs by several chemical compounds, acifluorfen (AF), methylacifluorfen (MeAF), hemin (HMN) and the haem breakdown products bilirubin-IX (BR) and biliverdin-IX (BV), some of which have previously been implicated in the inhibition of various forms of PPO and have been reviewed in Chapter 2.

The finding that *B. subtilis* PPO (Dailey et al, 1994a) has different inhibition characteristics from most other PPOs studied, led us to undertake these inhibitor investigations in an attempt to gain further information on the physicochemical nature of the enzyme active site and to characterise differences amongst the three forms of recombinant PPO.

Objectives

- To examine the potential inhibitory effects of various compounds such as AF, MeAF, BR, BV and HMN on the oxidation of protoporphyrinogen-IX by the human, *B. subtilis* and *M. xanthus* PPOs.
- To determine the kinetic constants, K_i , K_s , α ; and the mode of inhibition by these inhibitors.

Methods and Materials

Inhibitor assay preparation

Denatured *B. subtilis*, native human and *M. xanthus* recombinant PPOs were purified to apparent homogeneity as described in the previous chapters.

Renatured purified PPO was used for all the *B. subtilis* kinetic studies apart from a small pilot study in which semi-purified non-denatured enzyme was utilised for a comparison to show that K_m and V_{max} were unaffected by use of renatured enzyme.

The standard reaction mixture comprised an assay buffer (100 mM TrisHCl, 3 mM DTT, 1 mM EDTA, 0.1% Tween-20; pH 8.1 for human and *M. xanthus* and 8.7 for *B.*

subtilis), enzyme preparation, substrate and inhibitor. To ensure accurate determination of substrate oxidation, a corresponding blank was prepared for each and every assay reaction tube. This is described in detail in Appendices 8 and 9.

All inhibitors were dissolved in dimethylsulfoxide (DMSO) to a final solvent concentration of 2.5% in the reaction mixture. The blanks had the same concentrations of inhibitor or DMSO.

PPO assay in the presence of inhibitors

PPO activity was assayed in the presence of the various inhibitors by measuring the constant velocity formation of protoporphyrin-IX from protoporphyrinogen-IX. The reaction was measured at four regular time intervals over a period not exceeding 60 min. Return of fluorescence was directly proportional to time over the interval 10 – 40 min. and the slope of this line was used to calculate the reaction rate, nmol porphyrinogen-IX converted/ml of enzyme/h.

IC₅₀ values were determined for the inhibitors by measuring PPO activity at a range of inhibitor concentrations from 0 to 100 µM, using a substrate concentration of 10 µM. A double exponential curve of inhibitor concentrations vs PPO activity was fitted using a computerised software package (Enzfitter).

For determination of kinetic constants K_i , K_s and α , the substrate was added at 8 different concentrations in the range 0.5 to 15 µM. Four different inhibitor concentrations [I], within the range 0 to 10 µM (final concentration) were used for each substrate concentration. The velocity curves followed classic Michaelis-Menten kinetics. Both substrate and inhibitor concentrations were selected from pilot experiments to provide sufficient representative points on substrate-velocity plots to enable the accurate determination of the apparent Michaelis-Menten constants (K_{mapp}) and maximal velocities (V_{maxapp}), using the computerised Gauss-Newton iterative non-linear curve fitting procedure. The data represents the mean of at least three independent sets of results obtained from assays performed on different days with different enzyme preparations.

Derivation of constants

K_{mapp} and V_{maxapp} were determined from substrate-velocity plots, and the kinetic constants K_i , K_s and α ; and the model discriminations were determined from secondary replots of;

$$K_{mapp} / V_{maxapp} \text{ vs } [I], \text{ and } 1 / V_{maxapp} \text{ vs } [I]$$

where K_{mapp} and V_{maxapp} are the apparent or observed values in the presence of inhibitor and [I] = inhibitor concentration.

Definitions

- IC₅₀:- is the inhibitor concentration which at any fixed saturating substrate concentration (S^0), reduces the V_i (uninhibited) to $V_i/2$.

- K_i :- is equivalent to the inhibitor concentration required to double the slope of the double reciprocal enzyme velocity vs substrate concentration plot. Thus the lower the K_i the more effective the inhibitor.
- K_s :- refers to the dissociation constant of the enzyme-inhibitor complex.
- α :- is the factor by which K_s changes when the inhibitor occupies the active site of the enzyme.

Results

Inhibition of *B. subtilis* PPO

Bilirubin

The renatured *B. subtilis* PPO was inhibited by bilirubin (BR) (figure 6.1), yielding an IC_{50} of $10.8 \pm 1.8 \mu M$ ($n = 3$) with respect to protoporphyrinogen-IX as substrate. We have chosen to show this IC_{50} plot as an example. In further cases the data is presented, but the graphs are not shown.

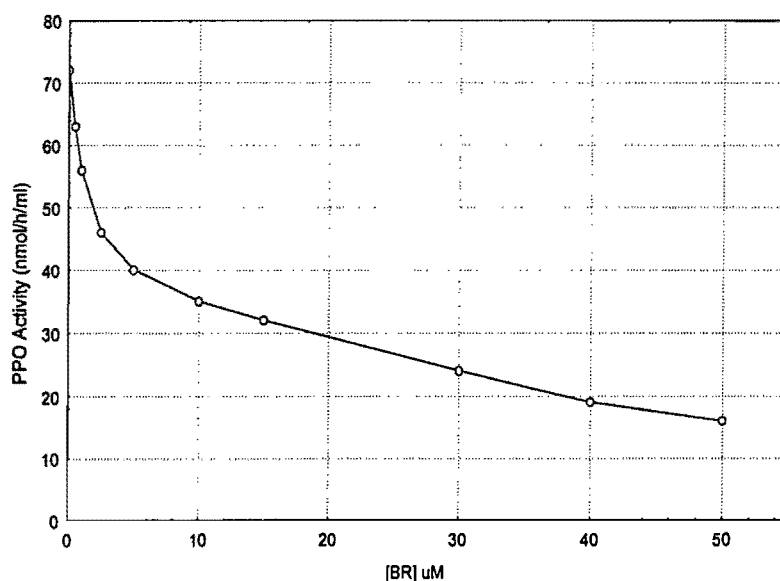


Figure 6.1: BR was an effective inhibitor of the *B. subtilis* PPO activity and a competitive mode of inhibition was obtained with respect to the oxidation of protoporphyrinogen-IX. $IC_{50} = 10.8 \pm 1.8 \mu M$ ($n = 3$).

Table 6.1 is an example of one of the 4 independently determined sets of inhibition data for the *B. subtilis* PPO in the presence of increasing [BR] showing increasing K_{mapp} values and an invariant V_{maxapp} which is indicative of competitive inhibition of the enzyme.

Table 6.1: Effect of [BR] on *B. subtilis* PPO activity. The increasing K_{mapp} relative to invariant V_{maxapp} at increasing [BR] indicated a competitive mode of inhibition.

[BR]	K_{mapp} (μ M)	V_{maxapp} (nmol/min/ml)
0	1.19	25.7
2.5	1.81	26.5
5.0	2.14	25.1
7.5	2.85	25.1
10.0	3.82	25.0

Analysis of the secondary replot of K_{mapp}/V_{maxapp} vs [BR] for the inhibition of the *B. subtilis* PPO yielded a K_i of $5.1 \pm 0.29 \mu$ M ($n = 3$). Secondary replot of $1/V_{maxapp}$ vs [BR] yielded a K_s of $4.16 \pm 0.4 \mu$ M ($n = 3$). An α value of infinity was obtained from this data confirming the competitive mode of inhibition of this enzyme by BR.

Table 6.2: The following values for K_{mapp}/V_{maxapp} and $1/V_{maxapp}$ were used in calculating K_i and K_s for the inhibition of the renatured *B. subtilis* PPO by BR from secondary replots (figures 6.2 and 6.3).

[BR] (μ M)	K_{mapp}/V_{maxapp}	$1/V_{maxapp}$
0	0.0463	0.039
2.5	0.0683	0.038
5.0	0.0853	0.040
7.5	0.114	0.040
10	0.153	0.040

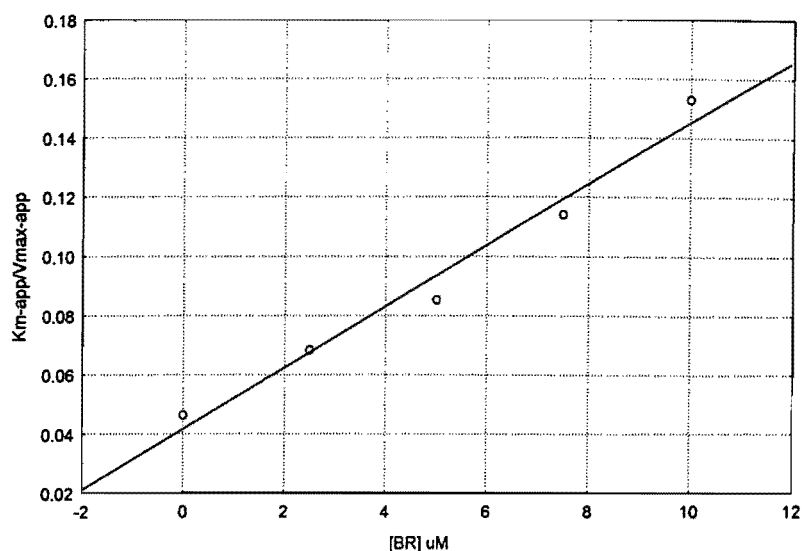


Figure 6.2: A secondary replot of $K_{m-app}/V_{max-app}$ vs $[BR]$ for the inhibition of the *B. subtilis* PPO by BR. A K_i of $5.1 \pm 0.29 \mu M$ ($n = 3$) was obtained.

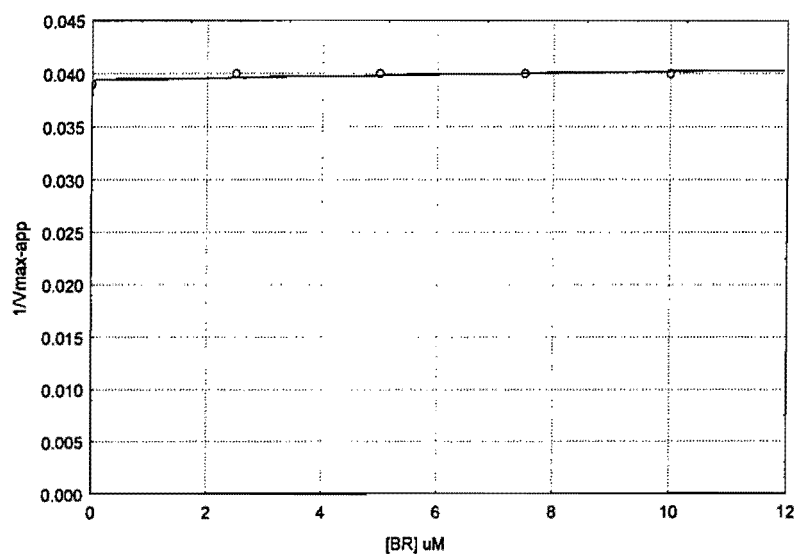


Figure 6.3: A secondary replot of $1/V_{max-app}$ vs $[BR]$ yielded a K_s of $4.16 \pm 0.4 \mu M$ ($n = 3$) for the inhibition of the renatured *B. subtilis* PPO.

The effect of BR on the enzyme was examined with both the renatured and non-denatured (native) enzyme preparations, and similar kinetic values and same mode of inhibition were obtained. Inhibition of the native enzyme by BR yielded IC_{50} of $11 \pm 0.2 \mu M$ ($n = 3$) and K_i of $5.08 \pm 0.4 \mu M$ ($n = 4$).

Biliverdin

BV was also an inhibitor of *B. subtilis* PPO and yielded an IC_{50} of $5.40 \pm 0.9 \mu M$ ($n = 4$) with respect to the oxidation of protoporphyrinogen-IX. As in the case of BR,

$V_{\max\text{app}}$ remained invariant in the presence of increasing [BV] whereas the K_{mapp} values increased, suggesting competitive inhibition.

A K_i of $4.03 \pm 0.66 \mu\text{M}$ and a K_s of $2.40 \pm 0.25 \mu\text{M}$ ($n = 4$) were obtained graphically from the secondary replots. An α value of infinity confirmed the competitive inhibition of *B. subtilis* PPO by BV.

Haemin

The inhibition of *B. subtilis* PPO by HMN was tested over a concentration range not exceeding $50 \mu\text{M}$ due to the difficulties in maintaining this inhibitor in solution at high concentrations. HMN yielded an IC_{50} of $10.2 \pm 1.4 \mu\text{M}$ ($n = 4$). Once again there was an increase in K_{mapp} , an invariant $V_{\max\text{app}}$ and an α value of infinity suggested a competitive mode of inhibition. A K_i of $2.31 \pm 0.2 \mu\text{M}$ and a K_s of 1.63 ± 0.21 was obtained with respect to the substrate, protoporphyrinogen-IX.

Acifluorfen and Methylaciflourfen

The *B. subtilis* PPO was not inhibited by $100 \mu\text{M}$ AF. At the same concentration MeAF reduced the enzyme activity minimally, by only 35%. Thus no further kinetic parameters for these two potential inhibitors were determined.

Summary

Table 6.3 summarises the kinetic inhibitory constants and model discrimination for *B. subtilis* PPO.

Table 6.3: Kinetic constants for the inhibition of *B. subtilis* PPO by BV, BR, and HMN, with respect to oxidation of protoporphyrinogen-IX. BR = bilirubin, BV = biliverdin, HMN = haemin, AF = acifluorfen, MeAF = methylacifluorfen.

INHIBITOR	IC_{50} (μM)	K_i (μM)	K_s (μM)	α	Mode of inhibition
BR	10.80 ± 1.80	5.10 ± 0.29	4.16 ± 0.40	∞	Competitive
BV	5.40 ± 0.90	4.03 ± 0.66	2.40 ± 0.25	∞	Competitive
HMN	10.20 ± 1.40	2.31 ± 0.20	1.63 ± 0.21	∞	Competitive
AF	No inhibition at $100 \mu\text{M}$				
MeAF	Minimal inhibition at $100 \mu\text{M}$				

Inhibition of *M. xanthus* PPO

An example of the inhibition of this enzyme by the various compounds, AF, MeAF, BV and HMN is illustrated in figure 6.4 (AF). The diphenylethers, AF ($IC_{50} = 0.26 \pm 0.04 \mu\text{M}$) ($n = 4$) and MeAF ($IC_{50} = 0.01 \pm 0.001 \mu\text{M}$) ($n = 4$) were highly effective inhibitors of this prokaryotic PPO compared to BV ($IC_{50} = 6.6 \pm 0.76 \mu\text{M}$) ($n = 4$) and HMN ($IC_{50} = 7.9 \pm 0.67 \mu\text{M}$) ($n = 4$) which inhibited to a lesser extent. BR did not inhibit this enzyme (up to $50 \mu\text{M}$ concentration and higher concentrations were not tested due to solubility problems).

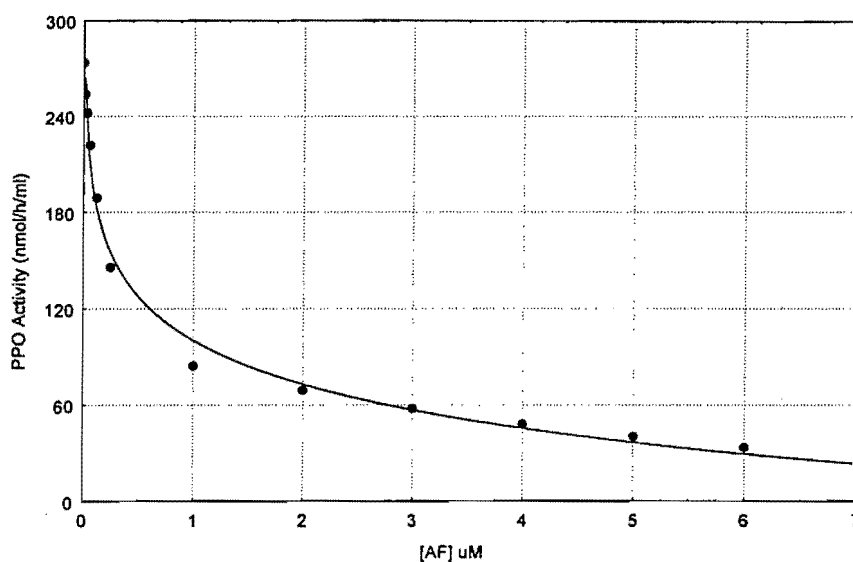


Figure 6.4: Effect of AF on *M. xanthus* PPO activity. AF was a strong effective inhibitor of the *M. xanthus* PPO activity. $IC_{50} = 0.26 \pm 0.04 \mu\text{M}$ ($n=4$) was obtained.

All the effective compounds were competitive inhibitors of this enzyme as observed by the increase in K_{mapp} values with V_{maxapp} values remaining relatively invariant in the presence of increasing concentrations of inhibitor (data not shown). Secondary replots of $1/V_{\text{maxapp}}$ against $[I]$ yielded α values of infinity for all the inhibitors, which confirmed the competitive mode of inhibition of the *M. xanthus* PPO by these compounds.

Table 6.4 summarises the IC_{50} , K_i , K_s and α values obtained for the inhibition of the *M. xanthus* PPO by AF, MeAF, BV and HMN, with respect to oxidation of protoporphyrinogen-IX.

Table 6.4: Kinetic constants for the inhibition of *M. xanthus* PPO by AF, MeAF, BV and HMN. The diphenylethers (AF and MeAF) were the strongest inhibitors of this enzyme.

Inhibitor	IC ₅₀ (μM)	K _i (μM)	K _s (μM)	α
AF	0.26 ±0.040	0.022 ±0.0020	0.041 ±0.050	∞
MeAF	0.01 ±0.001	0.003 ±0.0002	0.003 ±0.001	∞
BV	6.60 ±0.760	0.650 ±0.0900	0.240 ±0.020	∞
HMN	7.90 ±0.670	0.330 ±0.0400	0.530 ±0.020	∞
BR	No inhibition at 50 μM			

Inhibition of human PPO

This enzyme, like the *M. xanthus* PPO, was also more sensitive to the DPEs, AF (IC₅₀ = 4.8 ±0.28 μM) (n = 3), and MeAF (IC₅₀ = 0.17 ±0.015 μM) (n = 3) than to the tetrapyrrole compounds BV (IC₅₀ = 31 ±3 μM) (n = 3) and HMN (IC₅₀ = 20.81 ±3.1 μM) (n = 3). All the results were calculated from at least three independent sets of data from different assays, using different enzyme preparations. In all cases, and in particular for AF the inhibition was several fold less than for the *M. xanthus* form. Because of the poor inhibition of the human PPO by BV and HMN no further kinetic studies were performed with these compounds. Similarly to the *M. xanthus* form, the human enzyme was not inhibited by bilirubin up to 50 μM.

Figure 6.5 gives an example of the inhibition of human PPO by the DPE's. The IC₅₀ for AF was 5.0 ±0.9 μM (n = 3).

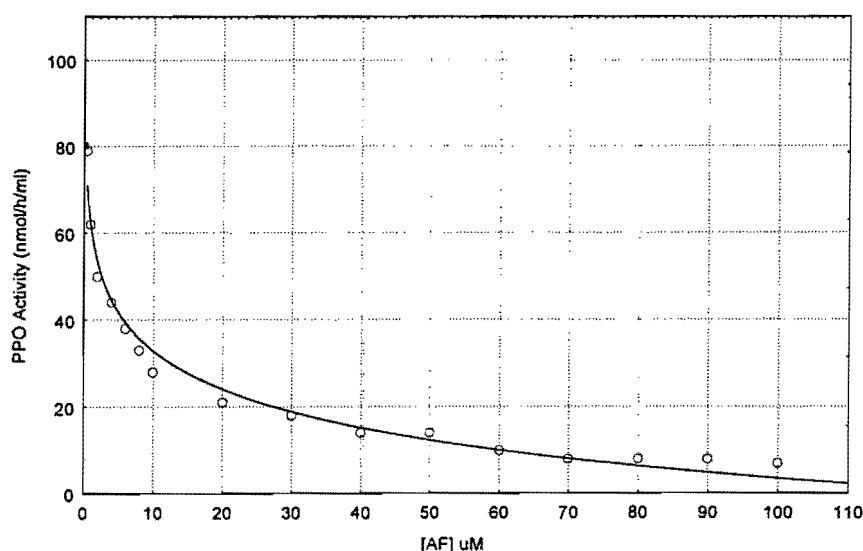


Figure 6.5: Effect of AF on human PPO activity. AF was a strong effective inhibitor of the human PPO activity yielding an IC₅₀ of 4.8 ±0.9 μM (n = 3).

Table 6.5 summarises the IC_{50} , K_i , K_s and α values obtained for the inhibition of human PPO by AF and MeAF. As BV and HMN were poor inhibitors, further kinetic values were not determined.

Table 6.5: Kinetic constants for the inhibition of human PPO by AF, MeAF, BV and HMN. The diphenylethers (AF and MeAF) were the strongest inhibitors of this enzyme. $n = 3$ for all the values.

Inhibitor	IC_{50} (μM)	K_i (μM)	K_s (μM)	α
AF	4.80 ± 0.28	0.240 ± 0.050	0.64 ± 0.10	∞
MeAF	0.17 ± 0.015	0.019 ± 0.001	1.73 ± 0.08	∞
BV	31.00 ± 3.00			
HMN	20.81 ± 3.10			

Discussion

In this study the effects of acifluorfen, methylacifluorfen, bilirubin, biliverdin and hemin were investigated on the activity of the purified recombinant PPOs of human, *B. subtilis*, and *M. xanthus*. In many cases these compounds were shown to be inhibitory which is possibly based on their electronic and structural similarities to the substrate molecule. Indeed, all of the above compounds which effectively inhibited the enzyme activity were competitive inhibitors with respect to the substrate, protoporphyrinogen-IX, indicating that they were binding very close to, or overlapping the enzyme catalytic site.

Indeed, inhibition by AF, of maize etioplasts and potato mitochondria (Camadro et al, 1991), as well as human placental and liver mitochondrial PPO (Corrigall et al, 1994) is of the competitive type. Mixed-type inhibition for the AF has been reported for yeast membrane (Camadro et al, 1991) and pig liver PPO (Corrigall et al, 1994). However, in yeast PPO, this mixed-type inhibition was subsequently shown to be due to partial hydrolysis of the enzyme and the mode of inhibition is in fact competitive (Camadro et al, 1994). Non-competitive inhibition of yeast PPO by both haemin and haem have been described (Poulson and Polglase, 1975) whereas competitive inhibition of mouse liver PPO by bilirubin has been illustrated (Ferreira and Dailey, 1988). Inhibition of human liver PPO by haem has been reported (Camadro et al, 1985).

Our data indicates clear interspecies behavioural differences. It is interesting that, in contrast to PPO from the prokaryote *M. xanthus* and other eukaryotes (Camadro et al, 1991, Corrigall et al, 1994, Dailey and Dailey, 1996a, 1996b) including human, *B. subtilis* PPO is not inhibited at $100\mu M$ acifluorfen and is only slightly inhibited by methylacifluorfen at this concentration. Methylacifluorfen is normally a more potent inhibitor of PPO than acifluorfen (Camadro et al, 1991) and was demonstrated in our results for the *M. xanthus* and human PPO. Furthermore a considerable range of inhibition by both AF and MeAF in the three species examined, was noted.

The lack of inhibition by these DPE's is not limited to *B. subtilis*. Two other prokaryotic PPOs, *E. coli* and *B. japonicum* were previously shown to be resistant to inhibition by the DPE's (Jacobs et al, 1990). Our findings suggest that these are not simply general differences between prokaryotic and eukaryotic forms of PPO as indicated by the different inhibitor profiles obtained for the *B. subtilis* enzyme and that of *M. xanthus*. Interestingly, the *M. xanthus* PPO behaved like the eukaryotic PPOs, being strongly inhibited by both AF and MeAF. It is possible that *B. subtilis*, *E. coli* and *B. japonicum* PPO exhibit conformational differences compared to the other PPOs making a good fit into the active site, for these DPEs, more difficult.

The PPOs of *M. xanthus* and *E. coli*, both Gram negative prokaryotes, behave differently towards the DPE's in that one was strongly inhibited and the other was resistant to inhibition. It therefore unlikely that differences in the behaviour of prokaryotic PPOs towards the DPE's are simply a reflection of the Gram +/- status.

Further differences were noted. Specifically BR inhibited *B. subtilis* PPO but was ineffective in inhibiting both human and *M. xanthus* PPO. On the other hand BV and HMN inhibited all three forms of PPO to varying degrees, although weakly in some cases.

In *B. subtilis* PPO, the IC₅₀ of biliverdin is approximately half that of bilirubin but the K_i's are similar. The conformation of bilirubin is more convoluted due to the intramolecular hydrogen bonding between the hydrophilic -COOH and -NH groups making it more rigid compared to biliverdin. Although biliverdin has a double bond at the C(9)-C(10) methene bridge, rotation can still occur at C(10)-C(11) which may present the dipyrrole moiety to the active site more favourably.

Both human and *M. xanthus* PPOs were resistant to inhibition by bilirubin. Interestingly, bilirubin was previously shown to be a competitive inhibitor of the purified mouse PPO with a K_i of 25 μ M, with respect to protoporphyrinogen-IX as substrate (Ferreira and Dailey, 1988).

A recent study by Arnould and Camadro (1998) on yeast PPO showed that, besides the $\beta\alpha\beta$ (dinucleotide binding) motif, two main blocks of conserved amino acid sequence homology were present in several PPOs, one is a C-terminal domain and the other an N-terminal domain. Relevant to that study was the fact that both conserved sequence homologies were identified in the human and *M. xanthus* PPO sequences whereas in the *B. subtilis* PPO sequence the C-terminal domain homology was not present. Based on mutagenesis studies these conserved sequence homologies appeared to be playing a crucial role in substrate binding and catalytic activity of these enzymes. In this work 28 PPO sequences were retrieved from the GenbankTM and TIGR (The Institute for Genomic Research) databases (see Chapter 7 for details). CLUSTAL-W 1.4 alignment of primary protein sequences revealed presence of these domains in several different PPO species (figure 6.6). Whilst the N-terminal domain (figure 6.6 - domain A) appears to be conserved in all the PPO species, homology of the C-terminal domain (figure 6.6 - domain B) is lacking in several prokaryotic sequences which include the *mycobacteriaceae* (*M. leprae*, *M. tuberculosis*, and *C. diphtheriae*), *B. subtilis*, *B. anthracis* and *D. radiodurans*. Importantly, both these

domains were not identified in the human monoamine oxidase-B (MAO-B) which was analysed with these PPO sequences.

These differences at the primary structural level may shed light on our observations of the behaviour of PPOs towards different inhibitors used in this study. Specifically, PPOs containing both conserved sequence homologies appear to have greater affinity for the DPE inhibitors, whereas in the case of the *B. subtilis* PPO which contains only the N-terminal conserved sequence these compounds are not effective. Furthermore, the *E. coli* PPO which lacks sequence homology to any of the known PPO sequences (Sasarman et al, 1993) also behaved like the *B. subtilis* enzyme towards these compounds. This could also explain the broad substrate specificity of the *B. subtilis* PPO. It would be interesting to see how the other prokaryotic PPOs which lack the C-terminal domain homology would behave like the *B. subtilis* PPO.

Domain A

• <i>H. sapiens</i>	PR-GKEP- <i>DE</i> -----TVHS <i>FAQRR</i> L <i>GP</i> EVASL-AMDSL <i>CRGV</i> FAGNSRE <i>LSIR</i> -SC <i>FP</i> SL
• <i>M. musculus</i>	PR-GKEP- <i>DE</i> -----TVHS <i>FAQRR</i> L <i>GP</i> EVASL-AMDSL <i>CRGV</i> FAGNSRE <i>LSIR</i> -SC <i>FP</i> SL
• <i>D. melanogaster</i>	ASKKAKLE <i>DE</i> -----SIYS <i>FAERR</i> F <i>GKEI</i> ADY-AISPMIC <i>GICAG</i> DAREI <i>SVR</i> -FLMEGL
• <i>M-N. tabacum</i>	KNKKLSQVSD--SHES <i>VSGFF</i> Q <i>RHF</i> G <i>KEV</i> VDY-LIDPFVAGTCGGDPDS <i>LSMH</i> -HS <i>FP</i> EL
• <i>G. max</i>	KRSDPSNVCDENS <i>SVGR</i> FFER <i>HFG</i> KEVVDY-LIDPFVGGT <i>SAAD</i> PES <i>LSMR</i> -HS <i>FP</i> EL
• <i>A. thaliana</i>	RP-SPPG <i>REE</i> -----S <i>VEE</i> FVRRNL <i>GDE</i> VFER-LIEPFCS <i>GVYAG</i> DPSK <i>LSMK</i> -AA <i>FG</i> KV
• <i>C-N. tabacum</i>	RP-SPPG <i>HEE</i> -----S <i>VEE</i> FVRRNL <i>GGE</i> VFER-LIEPFCS <i>GVYAG</i> DPSK <i>LSMK</i> -AA <i>FG</i> KV
• <i>Z. mays</i>	RP-PPPG <i>REE</i> -----S <i>VEE</i> FVRRNL <i>GAE</i> VFER-LIEPFCS <i>GVYAG</i> DPSK <i>LSMK</i> -AA <i>FG</i> KV
• <i>S. tuberosum</i>	RP-SPPG <i>YEE</i> -----S <i>VEE</i> FVRRNL <i>GAE</i> VFER-LIEPFCS <i>GVYAG</i> DPSK <i>LSMK</i> -AA <i>FG</i> KV
• <i>C. intybus</i>	RP-SPPD <i>REE</i> -----S <i>VEE</i> FVRRNL <i>GDE</i> VFER-LIEPFCS <i>GVYAG</i> DPSK <i>LSMK</i> -AA <i>FG</i> KV
• <i>S. cerevisiae</i>	KS-PHPGQ <i>DE</i> -----S <i>VES</i> ICDR <i>RF</i> GNNYISNNMISALL <i>RGIY</i> GD <i>DV</i> SL <i>LSAK</i> -RT <i>FK</i> KI
• <i>S. pombe</i>	SK--RDST <i>DE</i> -----S <i>VGS</i> FMRR <i>RF</i> GKNVTDR-VMSAMING <i>IYAG</i> DLND <i>LSMH</i> SS <i>MF</i> GL
• <i>M. leprae</i>	LS-WEPGSD <i>P</i> -----A <i>MAEL</i> VAD <i>RFGE</i> QAVAR-LVD <i>PLL</i> GGV <i>YAG</i> SAAT <i>I</i> GLR-AGAPSV
• <i>M. tuberculosis</i>	FT-WQVGS <i>D</i> P-----A <i>VAD</i> LVAD <i>RFGE</i> QVVAR-SVD <i>PLL</i> SGV <i>YAG</i> SAAT <i>I</i> GLR-AAAPSV
• <i>P. freudenr.</i>	RR--LPDH <i>DV</i> -----A <i>GAF</i> LRQ <i>RLGD</i> GIVRR-FADPMVGG <i>IYAG</i> AGID <i>ELSD</i> -AVLPSL
• <i>C. reinhardtii</i>	INGAMPS <i>FEE</i> -----S <i>VEE</i> F <i>IR</i> NRNL <i>GDE</i> VFFR-LIEPFCS <i>GVYAG</i> DPSK <i>LSMK</i> -AA <i>FN</i> RI
• <i>D. radiodurans</i>	PK-ENTD <i>EDE</i> -----S <i>LAD</i> FI <i>IR</i> RF <i>GAE</i> AMN--FIVPLAAG <i>IYAN</i> PF <i>ELSM</i> K-AA <i>FP</i> QF
• <i>M. xanthus</i>	RA--PEGV <i>DE</i> -----S <i>LAA</i> FGRRL <i>GHR</i> ATQV-LLDAVQT <i>GIYAG</i> DVEQ <i>LSVA</i> -AT <i>FP</i> ML
• <i>B. subtilis</i>	AS-KTKD- <i>DQ</i> -----S <i>LGE</i> FFRR <i>RGDE</i> VVEN-LIE <i>PLL</i> SG <i>IYAG</i> DI <i>DKLS</i> LM-ST <i>FP</i> QF
• <i>B. anthracis</i>	RS-KPVS- <i>DQ</i> -----S <i>LGH</i> FFR <i>RLGNE</i> VVEN-LIE <i>PLL</i> SG <i>IYAG</i> DI <i>DEM</i> SLM-ST <i>FP</i> QM
• <i>S. aureus</i>	PT-QMQD <i>GDI</i> -----S <i>VGA</i> FFR <i>RLGNE</i> VLEN-LIE <i>PLM</i> GG <i>IYGT</i> DI <i>DKLS</i> LM-ST <i>FP</i> NF
• <i>A. aeolicus</i>	GV----DED <i>I</i> -----S <i>IAD</i> FVRE <i>HFG</i> E <i>EFL</i> NY-VVAPFIS <i>GVYAG</i> DPEK <i>LSLK</i> -HATPKL
• <i>T. ferrooxidans</i>	PHPTPDG- <i>EE</i> -----S <i>IAD</i> FVRR <i>RLGDE</i> ALTW-LVD <i>PFV</i> SGV <i>FAGN</i> PAR <i>LSVQ</i> -ATL <i>PRL</i>
• <i>S. coelicolor</i>	RDAELPRTEVG-DDVA <i>VGEY</i> VAA <i>RLGRE</i> VVDR-LVE <i>PLL</i> GGV <i>YAG</i> DAY <i>ISLR</i> -SAVPQL
• <i>C. diphtheriae</i>	AESDSMDWPQ---E <i>VT</i> L <i>GEV</i> LRER <i>F</i> GD <i>LD</i> VDH-VVSALQGGV <i>YS</i> STS <i>DDLS</i> LR-DTVPQL
• <i>C. muridarum</i>	KQ-----D <i>S</i> -----S <i>VEA</i> FFKR <i>HSS</i> TKLRTH-LLNPIN <i>LAMR</i> AGH <i>SHI</i> LS <i>SAQ</i> -MAYPEL
• <i>C. trachomatis</i>	KQ-----D <i>S</i> -----S <i>VEA</i> FFKR <i>HSS</i> SKLRRN-LLNPIS <i>IAIR</i> AGH <i>SHI</i> LS <i>SAQ</i> -MAYPEL
• <i>C. pneumoniae</i>	TQ-----D <i>S</i> -----S <i>VQD</i> FLKR <i>HSS</i> QNFTSY-ILD <i>PLIT</i> AI <i>RA</i> GH <i>SSI</i> L <i>STH</i> -MA <i>FP</i> EL
• <i>H.sapiens</i> -MAO	KAPLAE <i>WDN</i> ---- <i>MTM</i> KELL <i>DK</i> LC <i>WT</i> ES <i>AKQ</i> -LATLFVN <i>LCV</i> TA <i>ETHE</i> V <i>SAL</i> WFLWYVK

Figure 6.6 (A). CLUSTAL-W 1.4 alignment of PPO protein sequences from various species showing the organisation of the N-terminal domain which is thought to be involved in membrane anchoring of these proteins through a nonpolar plateau (Arnould and Camadro, 1998). The underlined regions contain the conserved residues which are written in bold italic.

Domain B

<i>H. sapiens</i>	V----- QGFGHLVP -SSE--DPGV LGIVYDS ----- VAFP EQDGSP---PGLRVTV
<i>M. musculus</i>	V----- QGFGHLVP -SSE--DPTV LGIVYDS ----- VAFP EQDGNP---PSLRVTV
<i>D. melanogaster</i>	Q----- DGFG LVPPVEK---LPL LG VIFDS-----CC FDMG -----ENTVLTV
<i>M-N. tabacum</i>	N--VKYP LEGFGV LVPSKEQ QHGLKT LGTLFSS -----MM FPDR --AP---NNVLYT
<i>G. max</i>	N--VKRP LEGFGV LVPSKEQ KNGLKT LGTLFSS -----MM FPDR --AP---SDLYLYT
<i>A. thaliana</i>	C-LIDGEL KGFGQLHP -RTQ--GVET LG TIYSS-----SL FPNR --AP---PGRILLL
<i>C-N. tabacum</i>	R-LVDGEL KGFGQLHP -RTQ--GVET LG TIYSS-----SL FPNR --AP---KGRVLLL
<i>Z. mays</i>	C-LIDGEL QKFGQLHP -RSQ--GVET LG TIYSS-----SL FPNR --AP---DGRVLLL
<i>S. tuberosum</i>	R-LVDGEL KGFGQLHP -RSQ--GVET LG TIYSS-----SL FPNR --AP---NGRVLLL
<i>C. intybus</i>	R-LIDG QLKGFGQLHP -RSQ--GVET LG TIYSS-----SL FPNR --AP---PGRVLLL
<i>S. cerevisiae</i>	D-----L QGFGY LVPSK SNK -NPGKL LG VIFDS VIERNFKPLFDK LSTNPALNKYTKVT
<i>S. pombe</i>	R----- GFGL LIPSCTPNNPNHV LG IVFDSE-----QNNPEN-----GSKVTV
<i>M. leprae</i>	P-----QNS GV LVA-SGE--RLRAKAVTLSS-----RKWGLQ--GD-----TQLVR
<i>M. tuberculosis</i>	P-----HCS GV LVA-GDE--SPHAKAITLSS-----RKWQQR--GD-----VALLR
<i>P. freudenr.</i>	P-----DS QGW LEADAGP---VSG LT ASS-----IK FAGR --AP---DGSVLMR
<i>C. reinhardtii</i>	RKASDG SVPGFGQLHP -RTQ--GITT LG TIYSS-----SL FPGR --AP---EGHMLLL
<i>D. radiodurans</i>	M-----HLHGLQVADEG---IEMKAITVHS-----AKLHGR--AP---EGHVLLR
<i>M. xanthus</i>	D----- GF GLVPAEEQ---RRML GA IHAS-----TT FPFR --AE---GGRVLYS
<i>B. subtilis</i>	H-----EGTGFVIS-RNS--DFAITACTWTN-----KKWPHA--AP---EGKTLR
<i>B. anthracis</i>	I-----DGTGFVVS-RNS--DYTITACTWTH-----KKW PHT --TP---EGKTLR
<i>S. aureus</i>	H-----DGTGFVIA-RTS--DTDITACTWTS-----KKW PFT --TP---EGKVLIR
<i>A. aeolicus</i>	R-----L SS ESGK---KRIL G AMFMS-----KL FPGR --AP---QKELLT
<i>T. ferrooxidans</i>	L-----D GF GLIPRVMG---LET LG VLFS-----TL FPGR --AP---AGQVLLT
<i>S. coelicolor</i>	P-----EGS GF LVPPVDG---HTIKASTFAS-----RKWGWIADED---PDLVVLR
<i>C. diphtheriae</i>	N-----SGI L VATDAE---VTAKAFTFSS-----KKW PHLG --Q---RGGALVR
<i>C. muridarum</i>	H-----GY GM LFA--DT---P LL GI V ENT-----EV FPNS --QQ---PSTTSL
<i>C. trachomatis</i>	H-----GY GM LFA--DT---P LL GI V ENT-----EV FPQ P--ER---PNTIVSL
<i>C. pneumoniae</i>	K-----GY GM LFA--DE---L PL LGIVWNS-----QI FPQA --TP---GKTIVSL
<i>H. sapiens</i> -MAO	H-----KARK LARLTKEER LKK LCELYAKVLGS -----LEALEP VHYEE ---KNWCEEQ

Figure 6.6 (B) Organisation of the C-terminal domain identified in a CLUSTAL-W 1.4 alignment of various PPO primary sequences. This region is thought to contain important residues of the enzyme active site which are involved in catalysis (Arnould and Camadro, 1998). Importantly, this domain is less conserved in some prokaryotic PPOs such as *B. subtilis* which demonstrate poor substrate specificity and lack of inhibition by the diphenyl ethers which are known PPO inhibitors (This work, and Dailey et al, 1994a). The *Mycobacteriaceae* (*M. leprae*, *M. tuberculosis*, and *C. diphtheriae*), *B. anthracis* and *D. radiodurans* also lacked the sequence homology which is characteristic of this domain. Regions of high homology are underlined and the conserved residues written in bold italic.

Concluding Remarks

Our data highlights differences in the inhibition of *B. subtilis*, *M. xanthus* and human PPOs in the presence of AF, MeAF, BR, BV and HMN. When present, the mode of inhibition was always competitive probably as a result of electronic and structural similarities to the substrate molecule.

These clear behavioural differences between the different forms of this protein appear not to reflect simple prokaryotic and eukaryotic diversification nor Gram positivity/negativity in the case of prokaryotic differences.

Primary structure variation clearly affects the ability of PPO to handle various substrates and it's susceptibility to various inhibitors (see chapters 5, 6). While it was not the aim of this thesis to investigate the effects of alterations in gene sequence arising from both naturally occurring mutants and/or engineered recombinant protein, inhibitor and substrate studies on these forms may well provide information on the identity of amino acid(s) involved in catalysis and/or substrate binding. This chapter provides clear evidence that such detailed kinetic studies are indeed feasible.

Chapter Seven

Phylogenetic Analysis of Protoporphyrinogen Oxidases Using Protein Sequences

Introduction

PPOs, like many other known FAD-containing proteins, have been shown to contain a conserved signature sequence referred to as the dinucleotide binding motif (Wierenga et al, 1986). Analysis of PPO sequences revealed that these proteins share conserved regions with mammalian monoamine oxidases (MAOs) A and B, as well as with plant and bacterial phytoene desaturases (PHDs), and a signature motif was identified which groups these proteins into a superfamily of FAD-containing enzymes (Dailey and Dailey, 1998). In that study growtree analysis put all PPOs in a cluster more closely linked to a cluster of bacterial PHDs and more distantly related to mammalian MAOs (A and B) and plant PHDs.

All living organisms have a pattern of relationship based on their evolutionary history. At the molecular level evolutionary change over time is represented by the appearance of different gene and protein sequences. The vast information obtained from gene and protein sequences is therefore very useful in inferring phylogenetic relationships among sequences and also among the organisms they come from (Creighton, 1993, Swofford and Olsen, 1996). Evolutionary divergence into different species has resulted into many variants of the same protein containing different amino acid sequences, which essentially perform the same biological function. Generally, the higher the level of organisation, the greater the molecular differences among species. The importance of using gene and protein sequences in reconstructing evolution is based on the realisation that similar sequences generally imply descent from a common ancestor (Creighton, 1993). On the whole evolution is a gradual process and thus, species that are genetically more similar are likely to have diverged from each other more recently than they did from less similar species (Ayala and Kiger, 1980).

Biochemical studies on human, *B. subtilis* and *M. xanthus* PPOs carried out in this work (see chapters 5 and 6) revealed interspecies differences in the behaviour of these proteins. Generally, *M. xanthus* PPO was found to behave more like the eukaryotic enzymes than other prokaryotic PPOs. Importantly, this enzyme was strongly inhibited by the diphenyl ether herbicides such as AF and MeAF which

strongly inhibit many PPOs whereas, the *B. subtilis* (chapter 5 of this work), *E. coli* and *B. japonicum* (Jacobs et al, 1990) PPOs were shown to be resistant to inhibition by these compounds.

In view of our interest in pursuing the interspecies characterisation of these proteins, we performed a phylogenetic analysis of several PPOs, from both prokaryotes and eukaryotes. Presently, the two major sources of data for determining phylogenetic relationships between species are molecular (e. g. nucleic acid or protein sequences) and morphological (e. g. structure and anatomy of parts of plants and animals, and fossil records). Phylogenetic analysis based on nucleic acid or protein sequences are generally similar to those constructed by classical taxonomy techniques, and these are becoming more accepted due to the vast amount of information provided by sequences (Creighton, 1993). This section of the work describes the phylogenetic analysis of PPO sequences from diverse species and the relevant results are then discussed.

Objectives

- To perform a phylogenetic analysis of several PPOs, from both prokaryotes and eukaryotes using the available amino acid sequences of these proteins, in trying to establish their evolutionary relationship.

Methods and Materials

Identification and Retrieval of PPO Sequences from Genbank™ and TIGR Databases

Database searches of Genbank™ and TIGR (The Institute for Genomic Research) were performed by BLASTP method (Altschul et al, 1997) using the human (Dailey and Dailey, 1996b), *B. subtilis* (Hansson and Hederstedt, 1992) and *M. xanthus* (Dailey and Dailey, 1996a) PPO protein sequences. Identified PPO sequences were retrieved and kept in one file for multiple sequence alignment. The *Hem K* sequences and their homologs, originally thought to be PPO proteins (Nakayashiki et al, 1995), which were also hit during the blast search, were not included in the group as recent studies have revealed that these proteins do not have PPO activity (Le Guen et al, 1999).

Putative PPO sequences, which showed significant similarity to known PPO sequences, such as having the dinucleotide binding motif and other conserved regions, were identified and included in the analysis group. These included new sequences which have not yet been characterised, such as the 'PPO' sequences of *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Staphylococcus aureus*, and

Thermobacillus ferrooxidans which were identified in the unfinished genomes in the TIGR microbial genome database by TBLASTN method (Altschul et al, 1990), using the *B. subtilis* PPO sequence for the search. Nucleic acid sequences of these proteins showed significant similarity to PPOs and were retrieved from the genome sequencing projects. These were converted to protein open reading frames (ORFs), using the Molecular Biology Shortcuts (MBS) translator at the Expasy Molecular Biology server (http://www.expasy.ch/tools#translate_dna.html). The sequences were read from the first nucleic acid residue in six phases that is, 3 phases from both orientations of DNA. A blast search was performed with the putative protein sequences in Genbank™ to determine the level of identity and similarity to other known PPOs. Selected positive matches were those with significant identity and/or similarity of 45 - 80% to the known PPO sequences.

Short fragments, eg. less than 100 amino acids were not included in subsequent analysis, even though some of them showed similarity to PPOs, because these would disrupt the phylogenetic analysis. The entire search process in these databases yielded 28 PPO sequences in all which were used in the phylogenetic analysis. The human MAO-B sequence, retrieved from Genbank™ was used as an outgroup for rooting the tree during phylogenetic analysis.

Multiple Sequence Alignment and Phylogenetic Analysis

Performing multiple sequence alignments is a computationally intensive task. In essence the sequences are written down underneath one another, and then shuffled to-and-fro until the best possible match between one sequence and the next is obtained (Parkin, 1979). A comparison of the 28 PPO sequences and the human MAO-B sequence was done using CLUSTAL-W 1.4 alignment of protein sequences and applying its default values for gap creation and extension penalties. Since mutations occur during evolution of proteins it is necessary to allow for some insertion and deletion when comparing sequences, and this increases the percent identity (Parkin, 1979, Pearson and Lipman, 1988, Subbiah and Harrison, 1989, Altschul et al, 1990, 1997). The gaps in the multiple sequence pileup are created by computer analysis in order to maintain regularity, and these are assumed to be representing insertion or deletion of amino acid residues.

Results and Discussion

A multiple sequence alignment was produced in order to predict which sequence positions were likely to correspond. This is the simplest and most commonly used criterion for building phylogenetic trees and is referred to as the maximum parsimony method (Farris, 1983, Subbiah and Harrison, 1989). The general criterion of this method is to identify the alignment that minimizes the number of genetic mutations needed to account for the differences between the sequences. Figure 7.1 shows a pileup alignment of the N-terminal region of the different PPO and human MAO-B sequences indicating, within the boxed area, the dinucleotide binding motif (Wierenga et al, 1986). This alignment also shows the extended N-terminal sequences in the plant PPOs thought to be presequences involved in membrane targeting and translocation (Narita et al, 1996, Lermontova et al, 1997).

```

B._subtili      -----
M._xanthus     -----
H._sapiens     -----
A._thalian     -----MELSLRPTTQSLLP-SFSKPNLRLNVYKPLRLR
C-N._tabac     MTTTPIANHNPNIETHQS-----SSSP-LAFLNRTSFIP-FSSISKRNSVNCNGWRTR
A._aeolicu     -----
S._tuberos     MTTTAVANHPSIFTHRSPLPSPSSSSSSPSFLFLNRTNFIP-YFSTSKRNSVNCNGWRTR
S._cerevis     -----ML
M-N._tabac     -----MA
C._pneumon     -----
M._leprae      -----
M._tubercu     -----
S._pombe_P     -----
Z._mays_[m     -----MVAATATAMATAASPLLNGTRIPARLRHRLSVR
G._max_PPO     -----MA
M._musculu     -----
P._freuden     -----MSTTDRVTPTPTV
S._coelico     -----
C._intybus     --MTSLTDVCSLNCCRS-----WSSLPPPVSGSLTSKNPRYLITYSPAHRKCNRWRF
C._reinhar     MMLTQTPGTATASSRRS--QIRSAAHVSAKVAPRPTPFVSPATAASPATAAARRTLHR
C._trachom     -----
D._radiodu     -----MSVRRLRWR
D._melanog     -----
C._muridar     -----
B._anthrac     -----
S._aureus      -----
T._ferroox     -----
C._diphthe     -----
H._sapiens     -----

```

Figure 7.1 CLUSTAL-W 1.4 pileup alignment of the N-terminal regions of different PPOs and the *H. sapiens* MAO-B sequences showing the dinucleotide binding motif within the selected area [box] (continued in next page).

B._subtili	--MSDG-----KHH	VVIIGGGITGLAAAFYMEKE-IKEKNLPLELTVE
M._xanthus	---MHMPR-----TTGMN	VAVVGGGISGLAVAHHLRSR--G-----TDAVLE
H._sapiens	-----MGRT	VVVLGGGISGLAASYHLSRA-PC--PP--KVVLVE
A._thalian	CSVAGGPTVGSSKIEGGGGTTITTD	CVIVGGGISGLCIAQALATK-HPDAAP--NLIVTE
C-N._tabac	CSVAKDYTVPSSAVDGG--PAAELD	CVIVGAGISGLCIAQVMSAN-YP-----NLMVTE
A._aeolicu	-----MDMRE	VVVIGAGISGLSTAYRLKKE--G-----VDVVVYE
S._tuberos	CSVAKDYTVPPSEVDGN--QFPELD	CVVVGAGISGLCIAKVISAN-YP-----NLMVTE
S._cerevis	LPLTKLKP-----RAK	VAVVGGGVSGLCFTYFLSKLRPD-----VEITLFE
M-N._tabac	PSAGEDKHS-----SAKR	VAVIGAGVSGLAAAYKLIH-GL-----NVTVFE
C._pneumon	-----MKR	AIIGAGISGLAAGWWLHKK-FP----QAEILVLD
M._leprae	-----M-----TSRS	YCVVGGGISGLTAAYRLR----VATGDDVAITLFD
M._tubercu	-----M-----TPRS	YCVVGGGISGLTSAYRLR----QAVGDDATITLFE
S._pombe_P	-----MS	IAICGGGIAGLSTAFYLARLIPK-----CTIDLVE
Z._mays_[m	CAAVAGGAAEAPASTGAR---LSAD	CVVVGGGISGLCTAQAALATR-HG--VG--DVLVTE
G._max_PPO	SSATDDNPR-----SVKR	VAVVAGVSGLAAAYKLKSH-GL-----DVTVFE
M._musculu	-----MGRT	VIVLGGGISGLAASYHLIRG-PS--PP--KVILVE
P._freuden	SGTDAPGPD-----ASHCH	LVVVGGGITGLAAAWQGMAR--G-----ARVSVVE
S._coelico	-----	-----IAGLAAHRLLEAGAR-----VTVLE
C._intybus	CSIAKDSPITPPISNEFN-SQPLLD	CVIVGAGISGLCIAQALATK-HASVSP--DVIVTE
C._reinhar	TAAATGAPTASGAGVAKTLDNVYD	VIVVGGGLSGLVTGQALAAQ-HK--IQ--NFLVTE
C._trachom	-----MKH	ALIVGSGIAGLSAAWWLHKK-FP----HVQLSILE
D._radiodu	EGGPMTNPN-----AHTLP	IIVVGAGMTGLAAAWELQQR--G-----VPYVLE
D._melanog	-----MT	TAVLGGGLSGLSAGYYLLRR-FG-----KPLTIYE
C._muridar	-----MKH	ALVVGSGIAGLSAAWWLHKK-FP----HMQLSILE
B._anthrac	-CKKKGGAC-----LRKK	VVIIGGGITGLTTMYHLQKD-IRDKNLPIDTLIE
S._aureus	-----KS	VAIIGAGITGLSSAYFL-----KQQDPNIDVTIFE
T._ferroox	-----EE	VIIIGGGISGLATAYFLRKRGWS-----PL-LLE
C._diphthe	-----	-----GLTAAYRLRDH-----DVSVFE
H.sapiens	----MSN-----KCD	VVVVGGGISGMAAAKLLHDS--G-----LNVVLE

In order to facilitate phylogenetic analysis the gaps and widely divergent sequence regions were removed from the full sequences, leaving only the more conserved regions. Recent work has shown that the quality of a phylogenetic analysis improves when the input consists only of the highly conserved regions in a sequence alignment (Grundy and Naylor, 1999). The resultant sequence alignment (figure 7.2) was used for constructing the phylogenetic tree of these proteins using the Phylogenetic Inference Package [PHYLP] (Felsenstein, 1993) (figure 7.3).

Figure 7.2 A pileup alignment of protoporphyrinogen oxidase amino acid sequences from different species showing the dinucleotide binding motif in the boxed region (gaps and divergent sequence regions have been removed to facilitate phylogenetic analysis). The bold region represents part of the signature sequence of a superfamily of FAD-containing enzymes previously identified by Dailey and Dailey (1998).

	1						50
<i>B. subtili</i>	gl aaafymll ve	asprv	gk	iq	tv	kk	gyii ergp dsfler kkspqlvkdg
<i>M. xanthus</i>	gl avahhlal le	ssarl	gga	vg	thal	gylv	eqgp nsfldr eparalaaan
<i>H. sapiens</i>	gl aasyhlvl ve	sserl	ggw	ir	sv	rggaif	elgp rgirpa galllllvseg
<i>A. thalian</i>	gl ciaqallv te	akdrv	ggn	ii	tree	gflw	eegp nsfqps dpmtmvvds
<i>C-N. tabac</i>	gl ciaqvmlv te	ardrag	gn	itt	ver	gylw	eegp nsfqps dpmtmavdcg
<i>A. aeolicu</i>	gl stayrlvv ye	kddrig	gt	ih	tv	kegylf	evga qtilad qevidflkeg
<i>S. tuberos</i>	gl ciakvilv te	ardrag	gn	itt	ver	gylw	eegp nsfqps dpmtmavdcg
<i>S. cerevis</i>	gl cftyflil fe	sqnrt	ggw	iy	scnt	piml	ekgp rtlrgv sdgmdtlkdg
<i>M-N. tabac</i>	gl aaayklvv fe	aegkag	gk	lr	sv	sggliw	dega ntmtes egdtflidsg
<i>C. pneumon</i>	gl aagwwliv ld	keayag	gf	vr	tes	pgfsf	dlgp kgfltr gdgkcliheg
<i>M. leprae</i>	gl taayrlil fd	pgdrl	ggv	lr	tec	vgqpm	dlga eafllr rpepallaeg
<i>M. tubercu</i>	gl tsayrlil fe	padrl	ggv	lr	teh	igqpm	dlga eafvlr rpepallaeg
<i>S. pombe</i>	gl stafylil ye	kgprl	ggw	lq	sv	kitvlf	eqgp rtlrpa gvaldliskg
<i>Z. mays</i>	gl ctaqaalv te	ararp	ggn	itt	ver	gylw	eegp nsfqps dpvtmavds
<i>G. max</i>	gl aaayklvv fe	aegragr		lr	sv	sggliw	dega ntmtes eiekglidag
<i>M. musculus</i>	gl aasyhlvl ve	gskrl	ggw	ir	si	rggaif	elgp rgirpa galllllvseg
<i>P. freuden</i>	gl aaawqgvv ve	sddhf	ggk	vv	tdrr	gflv	eqgp dsfvay rpalklieeg
<i>S. coelico</i>	gl aaahrlvv le	asgrv	gk	ll	pge	igvr	dlga esmlar rpevglarag
<i>C. intybus</i>	gl ciaqaalv te	ardrv	ggn	ist	ver	gylw	eegp nsfqps damtmavds
<i>C. reinhar</i>	gl vtgqalfv te	arerv	ggn	it	sm	sggyvw	eegp nsfqpn dsmqiavds
<i>C. trachom</i>	gl saawwlli le	kesrs	ggl	iv	tek	qgfs	l nmgp kgfvla hdglhliqsg
<i>D. radiodu</i>	gl aaawelyl le	agdsf	ggk	vq	sert	eflv	ekaa dafilg kpwqlaqeg
<i>D. melanog</i>	gl sagyylli ye	asprv	ggn	vr	sen	rn	fif esgp rtirpv geplelvedk
<i>C. muridar</i>	gl saawwlli le	kesrp	ggl	vv	tek	qglff	nmgp kgfvvs hdglhliqsg
<i>B. anthrac</i>	gl ttmyhtl ie	asgkl	gk	iq	tv	rkgti	ergp dsflar kesarlvekeg
<i>S. aureus</i>	gl ssayflvi fe	asnpr	gk	iq	sy	rkgy	mi elgp esylgr ktitelakdg
<i>T. ferroox</i>	gl atayflll le	agaap	ggn	lq	sr	qegy	lr dmgp nslmlk gripewlreq
<i>C. diphthe</i>	gl taayrlvv fe	atdrig	gk	lh	tv	afsgpt	dmga eaylaf redtaffkeg
<i>H. sap. MAO</i>	gm aaakllvv le	ardrv	ggr	ty	tl	rn	kyv dlgg syvgpt qnrlrlakeg

	51						100
<i>B. subtili</i>	lelvn	rtlhp	mkgav	mg	ipt	klfsl	sgkar aamdfilpas slgeffrrrv
<i>M. xanthus</i>	ledpa	akryv	yrgrl	rs	vpa	silpl	garlr vagelfsrra slaafgrhl
<i>H. sapiens</i>	ldpaa	qnrl	ygga	lhal	pt	gppfs	kplfw aglreltkpr tvhsfaqrll
<i>A. thalian</i>	lkdpt	apr	fv	l	ngkl	rpvp	s klmsiggkir agfgalgrp sveefvrrnl
<i>C-N. tabac</i>	lkdpn	apr	fv	l	kgkl	rpvp	s klmsipgklr agfgaiglrp sveqfvrnl
<i>A. aeolicu</i>	iesps	skryi	ykgrl	ipl	pm	slslk	tklk vlteifkrgv siadfvrehf
<i>S. tuberos</i>	lkdpa	apr	fv	l	kdkl	rpvp	g klmsipgklr agfgaiglrp sveqfvrnl
<i>S. cerevis</i>	keciad	kfl	dsdkl	vq	vp	pn splg	kglitg mmgewfrkks svesicdrf
<i>M-N. tabac</i>	lrslq	nkryi	angtp	vll	ps	nflst	gsklq mllepilwn svsgffqrhf
<i>C. pneumon</i>	lqdra	akrfv	yrghk	kist	wrkgl	llpsli	kdfrapcytq svqdfklr
<i>M. leprae</i>	lsysq	qrlhs	lpdt	vag	ips	slvdd	atvar igaeavrpls amaelvadr
<i>M. tubercu</i>	lsysq	qrlhp	lpqt	vvg	ips	slvdd	atlar idaeaarpft avadlvadr
<i>S. pombe</i>	iesps	akryi	ypdrl	ne	ips	spalr	pmppla mmlepfrksk svgsfmrrf
<i>Z. mays</i>	lkdpn	apr	fv	l	egkl	rpvp	s klmsipgklr aglgaiglrp sveefvrrnl

G._max_	lqisqhkryi	vngapllvpt	nllsaqskih	lifepfmwkr	svgrffferhf
M._musculu	lepaaqnrfl	yggtlhlpls	gppfskplfw	aglirellkpr	tvhsfaqrri
P._freuden	lsllsrglrp	magmgmvlpt	rvlswpdkir	agldlviprr	aigaflrqrl
S._coelico	lappstaasl	wrgalrpmpk	gaaalsgvls	eeglarierd	avgeyvaaarl
C._intybus	lkdptaprfl	lggdlkpvp	klmsfpgklr	agfgalgfrp	sveefvrrnl
C._reinhar	cedptaprfl	wegklrpvp	glmsipgkir	aglgaglin	sveqfirrnl
C._trachom	laspeakrfi	hnnktrkvsp	wkqnlplsfa	kdfarfpykq	sveaffkrhs
D._radiodu	lqraetklyf	lggellldfpq	nvlspeglrr	mldeqnvppk	sladfiirrf
D._melanog	lehvaarrml	ykqqlcmlpn	sppftkplyk	avlrdlftas	siysfaerrf
C._muridar	lsspaakrfi	hnnktrkvsp	wkqnlpltli	kdlfarpykq	sveaffkrhs
B._anthrac	lglvnnrlhk	msgsmmgip	qlfspigklr	agfdllmpsr	slghffrhr
S._aureus	lefaknklyp	iggsimgip	dlsiplgklr	agfdllkkpt	svgaffrar
T._ferroox	lenplagryv	lnrhrqpval	gllsirgrlr	llgepfqpph	siadfvrri
C._diphthe	lespsglsl	ynghlpmppm	nskgleklvs	aetaaridae	tlgevlrerf
H.sapiens	leihhvkkxy	prgpfppvwn	pwrtmddmgr	eipsdapwka	tmkelldklc

101

150

B._subtili	gdevveliep	llsgiyagdi	dklslmstfp	qfyqslilgm	kktgqfqtls
M._xanthus	ghratqllda	vqtgiyagdv	eqlsvaatfp	mlvkslilga	iragalstfd
H._sapiens	gpevasamds	lcrgvfagns	relsirscfp	slfqsillgl	llgwsqwslr
A._thalian	gdevfeliep	fcsگویagdp	sklsmkaafg	kvwksiiggt	fkaqtvgftr
C-N._tabac	ggevfeliép	fcsگویagdp	sklsmkaafg	kvwksiiggt	fkaqtvgftr
A._aeolicu	geeflnvvp	fisگویagdp	eklslkhatp	klyeslikaf	ikegklisfg
S._tuberos	gaevfeliep	fcsگویagdp	sklimkaafg	kvwksiiggt	fkaqtvgftr
S._cerevis	gnnyismisa	llrgiygddv	sllsakrtfk	kiyydnmrk	srsypmlgla
M-N._tabac	gkevvdldp	fvagtcggdp	dsismhhsfp	elwnsvilga	irsrgsfsl
C._pneumon	sqnftsildp	litairaghs	silsthmaf	elaksllrsy	lknrylasls
M._leprae	geqavalvdp	llggvyagsa	atiglragep	svaaaldcga	tslpvfgald
M._tubercu	gdqvvasvdp	llsgvyagsa	atiglragep	svaaaldrge	tsvpvfgald
S._pombe	gknvtdvmsa	mingiyagdl	ndlsmhsmfg	flaknitlgl	iratsmfafk
Z._mays	gaevfeliep	fcsگویagdp	sklsmkaafg	kvwksiiggt	iktqtvasfr
G._max	gkevvdldp	fvgttsaadp	eslsmrhsfp	elwnsiiga	lqsrqsfstf
M._musculu	gpevasamds	lcrgvfagns	relsirscfp	slfqsillgl	llgwsqwslr
P._freuden	gdgivrfdp	mvggiygagi	delsldavlp	slrdslaggr	asrspftrtlr
S._coelico	grevvdlvdp	llggvyagda	yrslrsavp	qlfetsltea	vrapvfmgie
C._intybus	gdevfeliep	fcsگویagdp	sklsmkaafg	kvwnsivgga	fkaqtvgftr
C._reinhar	gdevffliép	fcsگویagdp	sklsmkaafn	riwislvgga	iklqtvgftr
C._trachom	ssklrrllnp	isiairaghs	hilsaqmayp	eltrslrsy	lkdpylatlr
D._radiodu	gaeamnfivp	laagiyvanp	felsmkafp	qflasvikgs	ratpifgsfk
D._melanog	gkeiadaisp	micgicagda	reisvrflme	glfegvlkgt	liswamygk
C._muridar	stklrtllnp	inlamraghs	hilsaqmayp	eltrslrsy	lrdpylatlr
B._anthrac	gnevveliep	llsgiyagdi	demslmstfp	qmyqsislgm	rtlglifqtk
S._aureus	gnevleliép	lmggiygtdi	dklslmstfp	nfkeslikgm	kdegqfkqfk
T._ferroox	gdealtlvdp	fvsgvfagnp	arlsvqatlp	rlaasllrga	lratrlvsfr
C._diphthe	gddlvdvsa	lqggvyssts	ddslrldtvp	qlarvtitaa	vqssvfaafp
H.sapiens	wtesaklatl	fvnlcvtaet	hevsalflwy	vkqcttnggq	erkpviyidq

151

200

B._subtili	tglqtlveei	ekqlykgtkv	tklshyslel	dndadsvivt	aphkaaagml
M._xanthus	ggqlvlidal	aaslarvegl	aredgwrlii	eesvaqvula	apahatakll
H._sapiens	gglemplqal	ethllrgqp	cglslwkvs	rdeadhvisa	ipasvlsell
A._thalian	kglrmlpeai	sarlwklsgi	tklesynlty	etqsksvvmt	vpshvasgll
C-N._tabac	kglrmlpdai	sarlwklssi	tksekyhlty	etqsrsvvmt	vpsvvasnil
A._aeolicu	eglgelinal	aqklnvvlrm	rkfedfrldv	rgetksvvva	spaytssyll

<i>S._tuberos</i>	kglrmlpdai	cerlwklssi	tksekyllty	etrsrcivmt	vpsyvasnil
<i>S._cerevis</i>	ggletfpkiv	rnalnptvtqi	mkrpattigl	katfdhlrlt	itppkiakll
<i>M-N._tabac</i>	ggmqtltdai	ckdlsvlel	scsctwsiis	assfdavimt	aplcdvksmk
<i>C._pneumon</i>	psmgltlitti	qekltsvthi	dcspkacvtt	psfadmviyt	gplqqlpvll
<i>M._leprae</i>	ggyqvlidel	vrrswvaatv	vgldrwtlvd	dtsadgvila	vpaprlvrll
<i>M._tubercu</i>	ggyqvllldgl	vrrswvrarv	vqlerwvlrd	etqadavila	vpaprlarlv
<i>S._pombe</i>	egietitlsi	adelmpnvki	hlnkpktlvp	hklvdvngga	yeyvfanss
<i>Z._mays</i>	kglamlpnai	tsslwkltsi	tksdyyvley	etqaksvimt	ipsyvasnil
<i>G._max</i>	ggmqtltdtl	ckelekvltl	ayghdwsits	asdvavimt	aplynvkdik
<i>M._musculu</i>	gglevlpqal	hnhllsgqp	cgslwkvsl	gdeadhiisa	ipaselskll
<i>P._freuden</i>	gglgqlidal	vdqllvntsv	dllgrvhlsd	grpadvvlla	ggvassarll
<i>S._coelico</i>	ggigtppav	adsvapvtel	rrtaswriwa	gdhagavvva	vpagpaaell
<i>C._intybus</i>	kgqamlpnai	strlwkltsi	sklenynlty	etqtktivmt	vpsyvasdll
<i>C._reinhar</i>	kglkmlpdai	erniwlkvlsl	greadyglvy	dtfaravalt	apsyvadvlv
<i>C._trachom</i>	sgmgmltqal	hdklapvski	rqlanisls	pqtgdmliya	gsvhdlpscl
<i>D._radiodu</i>	dgmstlanav	aaklrlnptv	lavhptltg	geaasgvvlt	apawvaaptl
<i>D._melanog</i>	gglenlpktm	rkylqlsne	rnltfvrnmi	kdpvehvss	lpayklapl
<i>C._muridar</i>	sgmesliqtl	hdkltptvki	rqlsnisls	sqtgdiliya	gsvqdlpscl
<i>B._anthrac</i>	tglesivesl	elkmikgtri	ekvakyaatl	sneadvvva	sshkvlpmsf
<i>S._aureus</i>	hglssfieal	eqdvryntsv	ddiitykivy	ndvydgvlt	tphqvflnwf
<i>T._ferroox</i>	eglqvlplrv	antltptvdql	aysgdwqvg	gnqskrlvla	lpagaaarl
<i>C._diphthe</i>	ggyaelyeal	aeksafitgi	qwkdgftlkg	aetfdkviva	vpaptaaml
<i>H.sapiens</i>	trenvlvetl	nhemsaippt	lgmkilpmmr	nqikcivyyk	epfwrkkdyc

201

250

<i>B._subtili</i>	elpaishlkn	mhtsvanval	gfpegvqmeh	fvisfaitac	twkkwphakt
<i>M._xanthus</i>	ddalaalvag	iaapiavvhl	gfdaglpapd	flvprmlga	ihthfprfrv
<i>H._sapiens</i>	aeaaplalal	satavsavv	nlqyqahlpv	hlvppgvlgi	vyvafpeqlr
<i>A._thalian</i>	sesaanalsk	lyppvaavsi	sypkeirtec	qlhpvetlgt	iyslfpnrri
<i>C-N._tabac</i>	svaaadalsn	fyppvgavti	sypqeirder	qlhpvetlgt	iyslfpnrsv
<i>A._aeolicu</i>	sfsaseefdk	idppvvvvnv	gvegkfpkvr	llsskrilga	mflklfgrke
<i>S._tuberos</i>	svaaadalss	fyppvaavti	sypqeirder	qlhpvetlgt	iyslfpnrsv
<i>S._cerevis</i>	qnsllskllde	iqntiilvny	ylpnkvidad	ylvpgkllgv	ifplfdklyt
<i>M-N._tabac</i>	iakrgnplll	nfpevdyvpl	svvitfkren	vlvplktlgt	lfmmfpdrvy
<i>C._pneumon</i>	ygienlskrv	lpwnlssisl	gwhhafslpk	mlfalpllgi	vwqifpqatv
<i>M._leprae</i>	qiaprtvaaa	srsvassavv	alsvpdttfp	vlvalrakav	tlrkwlgtq
<i>M._tubercu</i>	giaprthaaa	rqvsassavv	alavpgtafp	vlvaphakai	tlrkwggrva
<i>S._pombe</i>	enliscpkme	tpssvyvvnv	yykdpvlpir	llipnhvlgi	vfqnpensk
<i>Z._mays</i>	ssdaadalrs	fyppvaavtv	sypkeirkec	qlhpvetlgt	iyslfpnrsv
<i>G._max</i>	itkrgtfppl	nfpevsyvp	svmitfkken	vlvplktlgt	lfmmfpdrly
<i>M._musculu</i>	aeaaplalril	stkavsvavv	nlqyraqclpv	hlvpptvlgi	vyvafpeqlr
<i>P._freuden</i>	lpaaaralag	ipasttivsl	awpvsfdvap	wleavsglta	ssikfagrsv
<i>S._coelico</i>	apaaaaelsa	veasmalvtl	ayrrsaaalp	flvphtikas	tfrkwgwilv
<i>C._intybus</i>	slgaadalsk	fyppvaavsi	sypkdiradr	qlhpvetlgt	iyslfpnrsv
<i>C._reinhar</i>	apaaaalgs	fdppvgavtl	syplsvreer	qlhpittlgt	iyslfpgrhm
<i>C._trachom</i>	ipetklikqt	tswdlscvsl	gwhasfpiph	mlfapllgi	vfevfqpti
<i>D._radiodu</i>	fpaasalvge	lqngsvavil	ayregfprdm	lqvaiemkai	tvaklhgrhv
<i>D._melanog</i>	qqhpslsaq	lspyvdlvv	nmqfplllkq	llvplpllgv	ifccfdmgtv
<i>C._muridar</i>	ipesalvkqt	tswdlscval	gwhsslplph	mlfapllgi	vfevfpnstt
<i>B._anthrac</i>	qykqfrffrn	iptsvanvam	afpkisqrdr	fvvsytitac	twkkwphktk
<i>S._aureus</i>	qdpafdyfkt	mdttvatvvl	afdekienth	fviatditac	twkkwftkv
<i>T._ferroox</i>	datlarelda	ippavgsisi	gfrrpvehpl	mlipletlgv	lftlfpggrqv
<i>C._diphthe</i>	apeasallkn	ikassavvgm	kfdsaglpdn	ilvavtakaf	tffkwphlga
<i>H.sapiens</i>	iidgeeapva	ytdtdtkpegn	yaaaimfilah	rltkcelyak	vlealepvwc

	251		300
<i>B._subtili</i>	llrayvgndi	inivledlkk	vmningpemt
<i>M._xanthus</i>	lyscmvgdal	aalareelka	lagvtapsft
<i>H._sapiens</i>	vtvmlggelf	qqraqeaaat	qlglkepshe
<i>A._thalian</i>	lllnyiggel	veavdrdlrk	mlikpnplkl
<i>C-N._tabac</i>	lllnyigsq	vevvdrrdlrk	mlikpkplvv
<i>A._aeolicu</i>	lltvflgeei	eniverelke	ilqidcidfm
<i>S._tuberos</i>	lllnyigsq	veavdrdlrk	mlikpkpfvt
<i>S._cerevis</i>	kvtamigevt	inavkdalnn	hlgisnagqw
<i>M-N._tabac</i>	lyttfvgtel	keivtsdlkq	llgaegptyv
<i>C._pneumon</i>	lslliegsea	hafaiaalse	ylningpdaf
<i>M._leprae</i>	lvrlsfgd	lawavsdlaa	vfdvtvpdv
<i>M._tubercu</i>	llrlsfgdq	lawaaddlvt	vfgvavpvdv
<i>S._pombe</i>	vtvmmggee	vnnalkalqh	tlkissptlt
<i>Z._mays</i>	lllnyigsel	veavdrdlrk	mlinstplvl
<i>G._max</i>	lyttfigdel	rkivtsdlrk	llgaegptfv
<i>M._musculu</i>	vtvmlggelf	qqraqeaaat	qlglkepshe
<i>P._freuden</i>	lmrvfvpdel	lsavidhvrp	llgvhgpglt
<i>S._coelico</i>	vlrtsvgagl	vavsrhdlae	atgltapvat
<i>C._intybus</i>	lllnyiggei	vdavdrdlrt	mlirrdpltl
<i>C._reinhar</i>	lllnyigeql	veqvdkdlrn	mvikpdprvv
<i>C._trachom</i>	vslllegeea	yafslaaise	ylqiytpqaf
<i>D._radiodu</i>	llrvffkqka	laeavhevar	lfgagqplwh
<i>D._melanog</i>	ltvmmgkqi	vdlatshvqk	mlqirepkfs
<i>C._muridar</i>	lslllegeea	yafslatise	ylqiytpqaf
<i>B._anthrac</i>	llrcyvgeel	vqlvledlrk	tmditepeft
<i>S._aureus</i>	lirayvgnel	vsivrrdlsq	mmtfkgpeft
<i>T._ferroox</i>	lltafigddl	latvlreigp	llgisgpvfs
<i>C._diphthe</i>	lvrasfgdll	vdalddlqt	itgfdglsei
<i>H.sapiens</i>	eeqysggrvl	rqpvddriyfa	gtetatymeg

	301		321
<i>B._subtili</i>	kelrealapg	vymtgasfeg	v
<i>M._xanthus</i>	aaidaalqpg	lhlgayayk	v
<i>H._sapiens</i>	esarqfltlp	ltlagasyeg	v
<i>A._thalian</i>	dtaksslteg	lflggnvvg	v
<i>C-N._tabac</i>	stakaamneg	lflggnvvg	v
<i>A._aeolicu</i>	nlaqemekpg	lfltggnwlyg	v
<i>S._tuberos</i>	gtaktalsdg	lflggnvvg	v
<i>S._cerevis</i>	eraerklqqt	vsvggmgfssr	p
<i>M-N._tabac</i>	daidkmekpg	lfyagnhrvg	l
<i>C._pneumon</i>	erilphlpqn	lkivggniaq	p
<i>M._leprae</i>	aevraglppt	lvvagsmdg	i
<i>M._tubercu</i>	aelraglppt	lavagsyldg	i
<i>S._pombe</i>	nslkswiegr	illtgswyng	v
<i>Z._mays</i>	eaakaalddg	lflggnvvg	v
<i>G._max</i>	qaidkiekpg	fffagnykkg	l
<i>M._musculu</i>	dsamqfltlp	ltlagasyeg	v
<i>P._freuden</i>	avvdstlapt	wavagsalhg	v
<i>S._coelico</i>	arvrehvapg	lavcgaaydg	v
<i>C._intybus</i>	dsakaalsqg	mflggnvvg	v
<i>C._reinhar</i>	dkarkaldqg	vhlggnvvg	v
<i>C._trachom</i>	qrllsklphn	ikivggnfag	p


```
D._radiodu  ariraalppn iqvagssytg v
D._melanog  eairnyiklp lsvcgaaaydg v
C._muridar  killsklppn ikivgqnfag p
B._anthrac  kklttfmepg iylagssyag s
S._aureus   rqiqahikpr lrvtgasfea v
T._ferroox  kridtlsapg lhfranwqgg v
C._diphthe  aaikdalpvg iyatgawadg v
H.sapiens   deiwqseppa qpitttfler p
```

Phylogenetic analysis of these PPO sequences (figure 7.2) was done using the maximum parsimony method (Farris, 1983, Subbiah and Harrison, 1989). When a tree is constructed it may be rooted, meaning that one of the sequences represents a common ancestor to all the others and this is used as the outside distant protein, or it may not be rooted, meaning that no common ancestor could be identified (Swofford and Olsen, 1996). Although the human MAO-B sequence was used to root the tree, phylogenetic analysis using maximum parsimony produces trees with no evolutionary root (Weston, 1994), and the human MAO-B sequence was used as an outgroup for the construction of this tree. The rooting of this tree with human MAO-B derives from the fact that, although MAOs share a region of similarity with PPOs, comprised of the signature sequence of FAD-containing enzymes identified by Dailey and Dailey (1998), PPOs are more closely related to each other than they are to MAOs.

The procedure for the PHYLIP package creates several possible trees and chooses the tree that minimizes the total number of mutations required throughout the sequences. Statistical analysis of the phylogenetic results was done using 'bootstrap' analysis (Felsenstein, 1988) and this showed that the resultant tree (figure 7.3) was statistically significant. Basically, the technique involves analysis of a sample of randomly perturbed data sets (100 in this case). In each perturbation the original characters (protein sequence positions) are randomly resampled with replacements and the new sequence alignments, of same length, are analysed as for the real original data.

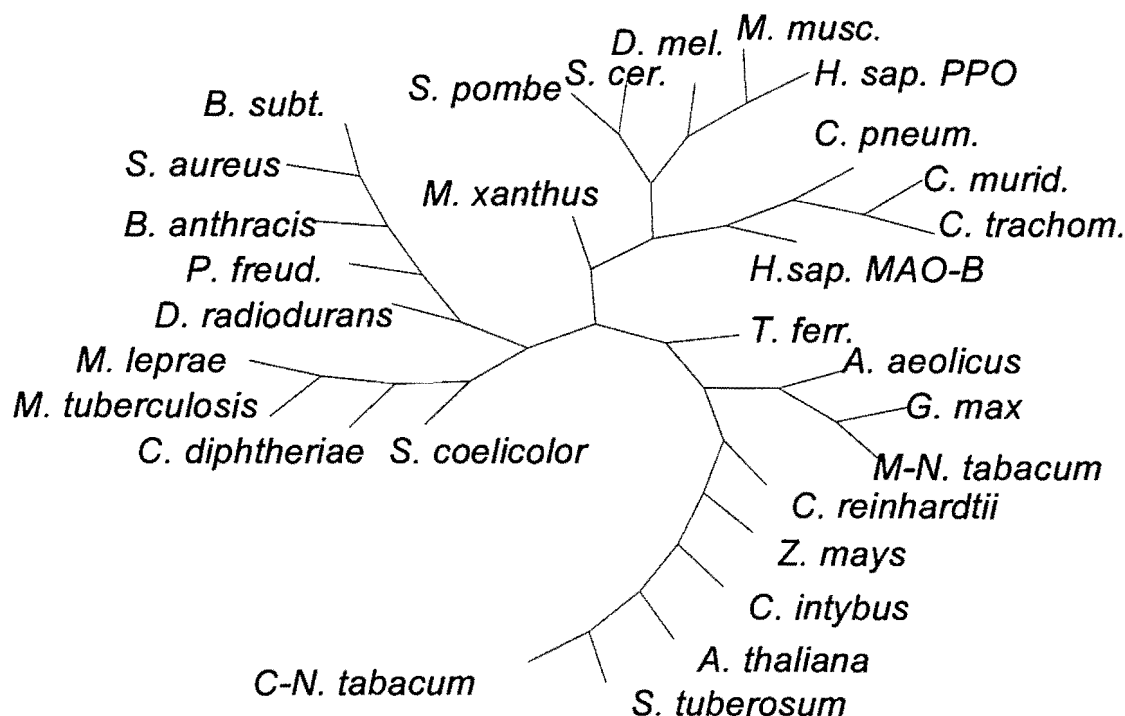


Figure 7.3 Growtree analysis of the different PPO sequences (figure 7.2) showing the phylogenetic relationship between these proteins.

The data showed mammalian PPOs clustered together with the insect protein from *D. melanogaster* (*D. mel.*) indicating a close relationship as would be expected. Interestingly, this cluster was more closely linked to that of yeast, *S. cerevisiae* (*S. cer.*) and *S. pombe*, PPOs than to plants as would normally be expected. Importantly, all plant PPOs were clustered together although they branched into two groups, apparently on the basis of the organelle in which the PPO isoform was located. For example, the *N. tabacum* (*C-N. tabacum*), *S. tuberosum* and *A. thaliana* chloroplast isoforms, and other closely related plant PPO sequences were grouped together, and these proteins share the extra N-terminal presequence. The mitochondrial (M-) isoforms and similar sequences formed the second group of plant PPOs. This suggests different ancestral origins of the proteins from these two organelles.

The green alga, *C. reinhardtii*, was clustered with plant PPOs (figure 7.3), as would be expected, and it was grouped with the chloroplast isoforms. Like these isoforms, it also had an extra N-terminal sequence (figure 7.2). Previously, molecular characterisation of the wild-type *C. reinhardtii* PPO showed that this protein had significant homology to the chloroplast PPO isoforms of *A. thaliana* (51%) and *N. tabacum* (53%), suggesting that it was also located in the chloroplasts (Randolph-Anderson et al, 1998).

Most of the bacterial PPOs were grouped together from a common branch, indicating a common ancestral origin but, they branched into two distinct groups. Interestingly, both Gram positive and negative organisms were present in both groups. For example, *P. freudenreichi*, a Gram negative rod was grouped with several Gram positive organisms, *D. radiodurans*, *B. anthracis*, *B. subtilis* and *S. aureus*. All the organisms belonging to the Gram positive *Mycobacteriaceae* family, *M. tuberculosis*, *M. leprae*, and *C. diphtheriae* were clustered together indicating a common ancestral origin.

Interestingly, the *M. xanthus* PPO shared a distant common branch with the eukaryotic (yeast and mammalian) PPOs. Biochemical studies previously showed that, in terms of its properties the *M. xanthus* PPO behaved more like the eukaryotic than prokaryotes PPOs (this work chapters 4 and 5, Dailey and Dailey, 1996a). These findings therefore confirmed the biochemical behaviour of this enzyme. The thermophilic bacteria, *T. ferrooxidans* (*T. ferr.*) and *A. aeolicus* also diverged from the general clustering of prokaryotic PPOs as they shared a distant clustering with the mitochondrial isoforms of plant PPOs. It seems therefore that these bacterial proteins share a common ancestral origin with these isoforms of plant PPOs. Generally, it seems that prokaryotic PPOs are quite distinct phylogenetically and this is similar to the findings on bacterial MAOs reported previously (Dailey and Dailey, 1998).

Concluding Remarks

The grouping of these proteins shows that the phylogeny based on these PPO sequences is similar in many respects to species phylogenies constructed by classical techniques using morphology and fossil records. Interesting observations from this study were the divergences of *M. xanthus*, *T. ferrooxidans* and *A. aeolicus* from other prokaryotes, and the close relationship of these organisms to eukaryotic proteins.

Chapter Eight

Immunocharacterisation of Human Protoporphyrinogen Oxidase

Introduction

Chapters 4, 5 and 6 reported on the biochemical characterisation of recombinant human, *M. xanthus* and *B. subtilis* PPOs. Their kinetic properties and inhibitor profiles revealed some inter-species differences. The phylogenetic analysis of PPOs from diverse species, based on 28 protein sequences, obtained from several protein databases is described in chapter 7.

Little is known of the tissue distribution (intra- and inter-organ) of PPO in humans. PPO activity has been described in human liver (Camadro et al, 1985, Corrigall et al, 1994, Li et al, 1989), placenta (Corrigall et al, 1994), skin fibroblasts (Brenner and Bloomer, 1980b), lymphocytes (Guo et al, 1991) and Epstein-Barr virus transformed lymphoblasts (Meissner et al, 1986). While all tissues are potentially able to synthesize haem, it is assumed that those primarily involved in haem production would demonstrate strong expression of various haem enzymes, including PPO. However, this data is lacking.

Earlier studies using subcellular fractionation methods showed that yeast and mammalian PPOs were associated with mitochondrial membranes and required detergents for solubilisation (Poulson and Polglase, 1975, Poulson, 1976). PPO activity was localised to the cytoplasmic side of the inner mitochondrial membrane (Deybach et al, 1985, Ferreira and Dailey, 1988). In subsequent studies, using specific labelled PPO ligands such as the tetrahydrophthalimide inhibitors, presence of this protein was demonstrated in subcellular fractions of mitochondria (Matringe et al, 1992b) and the *in vitro* translation and targeting of labelled PPOs into mitochondria and chloroplasts has been demonstrated in preparations of these organelles (Nishimura et al, 1995b, Lermontova et al, 1997). To date, no studies on the immunohistological localisation of PPO have been reported.

In view of our interest in determining the properties of PPOs, and the ability to produce antibodies against these proteins, we embarked on immunocharacterisation of human PPO. This section of the work reports on the immunohistological localisation of human PPO in a variety of organs, in an attempt to shed light on the intra- and inter-organ expression of this protein. It also reports on the subcellular localisation of PPO in human hepatocytes, in which the electron microscopy techniques of cryoultramicrotomy and immunogold labelling were adapted for use with human liver biopsy specimens.

Objectives

- To demonstrate intra- and inter-organ expression of PPO in a variety of human organs using immunohistological localisation methods and light microscopy.
- To determine subcellular location of human PPO in liver tissue sections using immunogold labelling techniques and electron microscopy.

Methods and materials

Antibody production

Human PPO was purified as previously described (see chapter 4). Purified PPO was blotted onto nitrocellulose paper (0.45 μm) (Schleicher and Schuell, Dassel West, Germany), dissolved with DMSO and used for the inoculation of New Zealand white rabbits (Kirsch et al, 1975), using approximately 100 μg of PPO per inoculation. The strength and specificity of the antibody were assessed by Western blotting (Towbin et al, 1979). For full details of the methodology see Appendix 10.

Light Microscopy - Determination of the expression of PPO in various human tissues

Tissue specimens

Surgically removed material was examined from liver, kidney, lung, testis, ovary, stomach, small and large intestine and placenta. Small intestine and colon specimens were used as representative tissue of small and large intestine, respectively. Only normal tissue was used in the study. All specimens were fixed in buffered formal-saline, embedded in paraffin wax and sections were cut at five microns. Five examples of each tissue were examined.

Immunohistochemistry

The immunoperoxidase technique was performed essentially as previously described (Campbell et al, 1991) except that the swine anti-rabbit immunoglobulins utilised were biotinylated and streptavidin conjugated to the horseradish peroxidase. Briefly, the tissue sections were dewaxed and incubated in 0.3% H_2O_2 in methanol to block endogenous peroxidase. The sections were exposed to normal swine serum (1/20) in PBS for 15 min followed by incubation with a 1/100 dilution of rabbit PPO antiserum for 60 min. The sections were exposed to biotinylated swine anti-rabbit immunoglobulins (1/250) for 30 min. After exposure to streptavidin conjugated to horseradish peroxidase (1/500) the specifically bound antibody was visualised using the substrates, 3,3-diaminobenzidine (0.5 mg/ml in PBS) and 0.01% H_2O_2 .

Counterstaining was with Meyers haematoxylin. The entire procedure was performed at room temperature.

Non-immune rabbit serum at the same dilution as the primary antibody was used as a negative control for each and every specimen studied. Pi glutathione S-transferase (GST) specifically stains bile ducts in human liver (Campbell et al,1991) and a liver section thus stained was included in every run as a positive control. The immunostaining was evaluated by an arbitrary semiquantitative scale as being strong, moderate, weak or negative. See Appendix 11 for full details.

Electron microscopy - Determination of the sub-cellular distribution of PPO in human hepatocytes

Liver tissue specimens

Fresh human liver biopsy tissue was obtained, with informed consent, from patients who were undergoing routine, follow-up investigation at the Liver Clinic at Groote Schuur Hospital, Cape Town. Small tissue pieces ($0.5\text{--}1\text{ mm}^3$) were used for the immunohistological investigations and these were cut from biopsy specimens taken for the routine histopathological diagnosis of these patients. The methods of cryoultramicrotomy and immuno-gold labelling were optimised for use with fresh human liver biopsy specimens. See Appendix 11 for full details.

Specimen preservation and cryoultramicrotomy

Considerable time was spent achieving the desired preservation of tissue ultrastructure and antigenicity. The best preservation was obtained when the small liver tissue pieces were immediately transferred into a fixative (4% paraformaldehyde, 0.1% glutaraldehyde, in PBS buffer pH 7.4), and fixed for at least 6h, followed by cryoprotection in sucrose. The fixed tissue specimens were gradually infiltrated overnight with sucrose up to a final concentration of 2.3M, and then rapidly frozen in liquid propane at -180°C . Thereafter very thin (120 nm) microtome sections were cut at -120°C and transferred onto formvar-covered carbon-coated nickel grids for the immunolabelling procedure.

Immunogold labelling

For immunohistological detection and electron microscope visualisation of PPO, an immunogold labelling protocol (Horrisberger and Rosset, 1977, Pollack and van Noorden, 1987), which effectively attaches 5 nm gold particles to the PPO bound primary antibody in the liver sections, was used.

The tissue sections on nickel grids were blocked for non-specific antibody reactions with 2% gelatin and 20 mM glycine in PBS pH 7.4. The primary PPO antibody reaction was then performed using 1/50 dilution of rabbit serum in which PPO

antibody had been raised. After rinsing the sections the antibody bound to PPO was then labelled with a goat antirabbit complex containing the 5 nm gold particles for electron microscope visualisation. After stabilisation of the gold complex with 1% glutaraldehyde, and silver enhancement for 4 min (Hainfeld and Furuya, 1995), the sections were stained with uranyl acetate/methyl cellulose and dried before examination with a Zeiss EM 109 transmission electron microscope. Several micrographs of the experimental and control sections were taken. Normal rabbit serum (1/50) dilution replaced the PPO antibody in the controls.

An initial study using transformed *E. coli* cells which overexpress recombinant human PPO was performed as further confirmation of positive reactivity of the antibody and visualisation of human PPO. The cells were grown overnight and harvested for labelling as described above for the liver cells. The cells were also fixed in the same solution and then mounted onto low melting point agarose for cryosectioning. Further processing of the bacterial cells was done as described for the liver cells.

Results

Western blotting

The results of Western blotting are shown in figure 8.1. Specificity of the antibody to human PPO is demonstrated by the single band in Figure 8.1A lanes 1 and 2. No bands were apparent in the non-immune control, Figure 8.1B lanes 1 and 2.

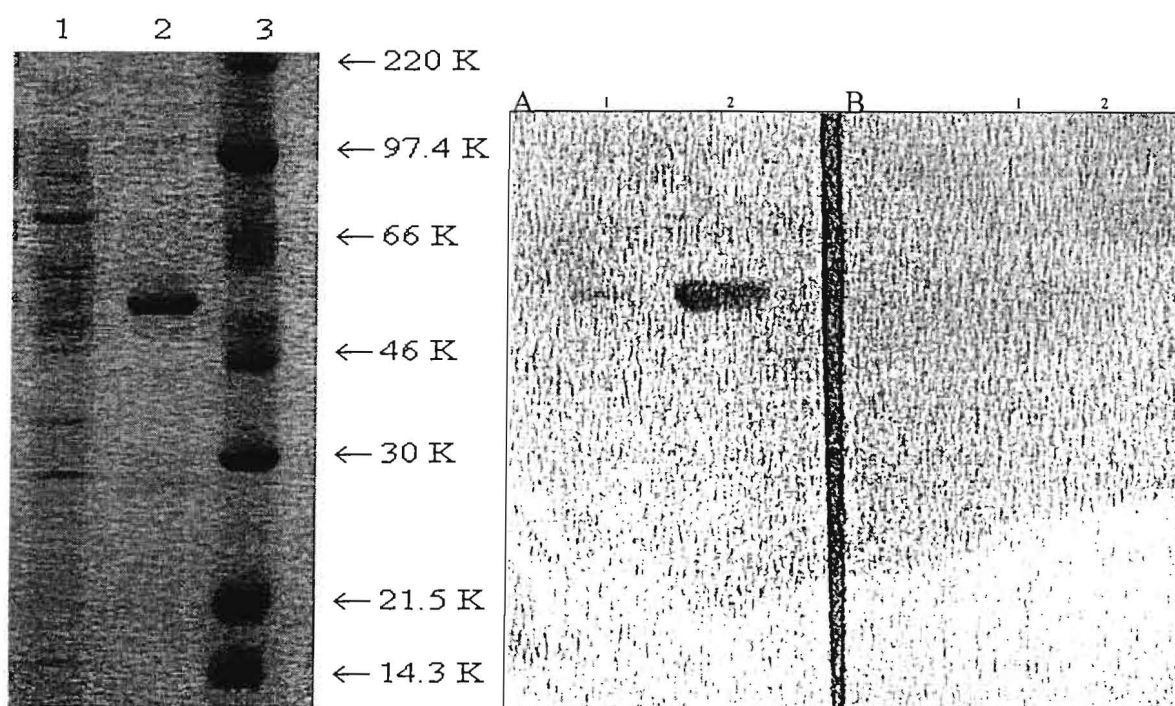
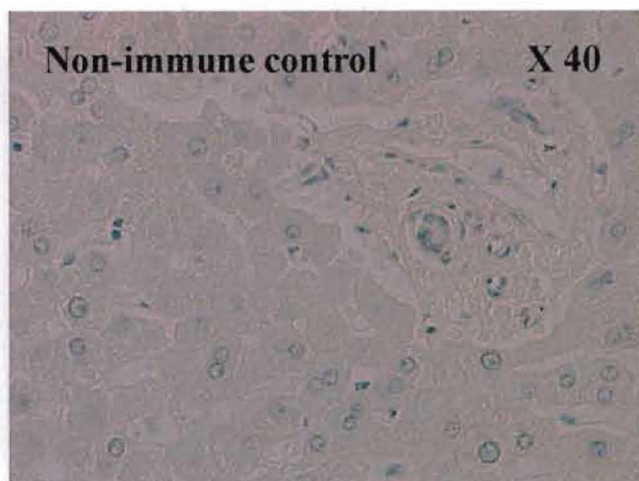
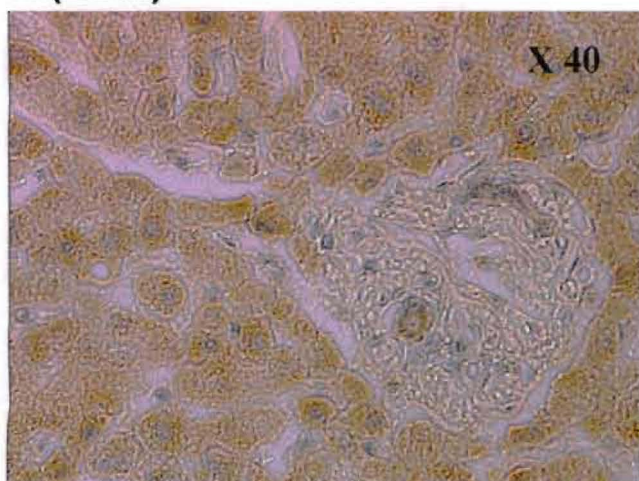


Figure 8.1 Western blot showing specificity of the polyclonal antibody to human PPO. On the left is the SDS -polyacrylamide gradient gel (7.5-17.5%) showing in lane 1 = sonicate of *E. coli* cells expressing recombinant human PPO with a wide spectrum of proteins, lane 2 = purified human PPO, and lane 3 = molecular weight markers. On the right, A and B = Western blots of identical gels on Nitrocellulose paper, A = incubated with the human PPO antibody and B = incubated with non-immune normal rabbit serum. The single positive band in A, lane 1 shows specificity of the antibody to human PPO in the presence of the wide spectrum of proteins from the sonicate of *E. coli* cells.

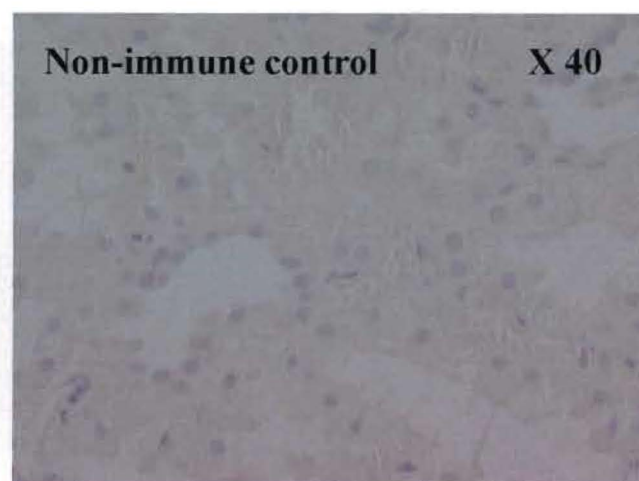
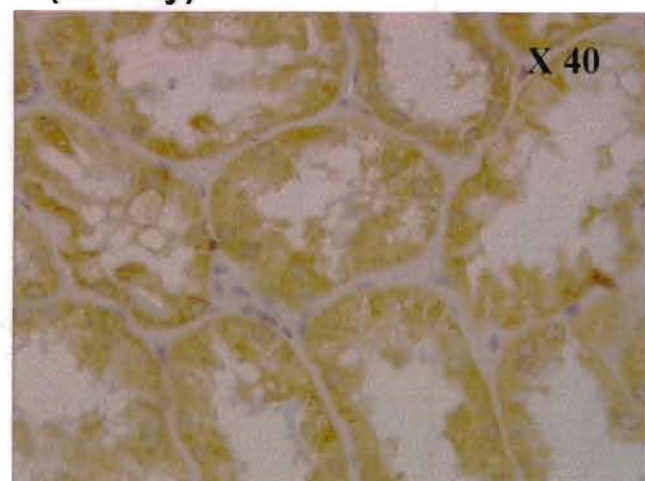
Light Microscopy - Expression of PPO in various human tissues.

The five livers examined showed a strong staining of the cytoplasm of the hepatocytes, but not the nuclei. Kupffer cells were negative but staining of the bile ducts was noted. No staining for PPO was apparent in all the non-immune controls (figure 8.2 A).

A (Liver)



B (Kidney)



C (Lung)

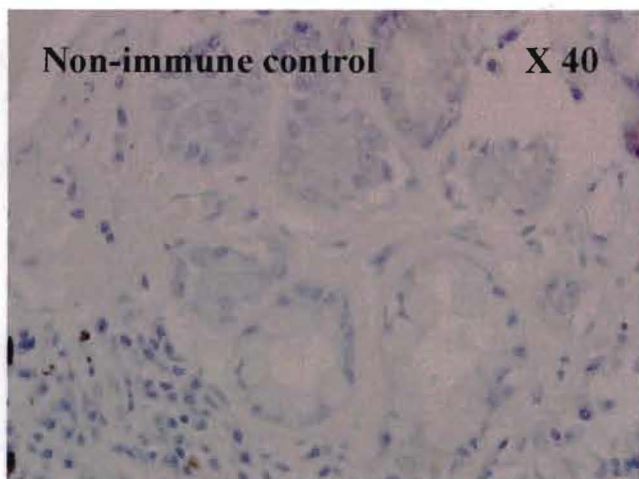
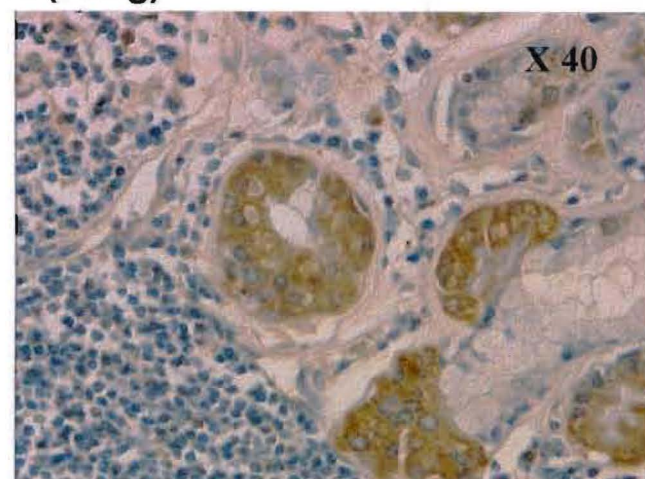
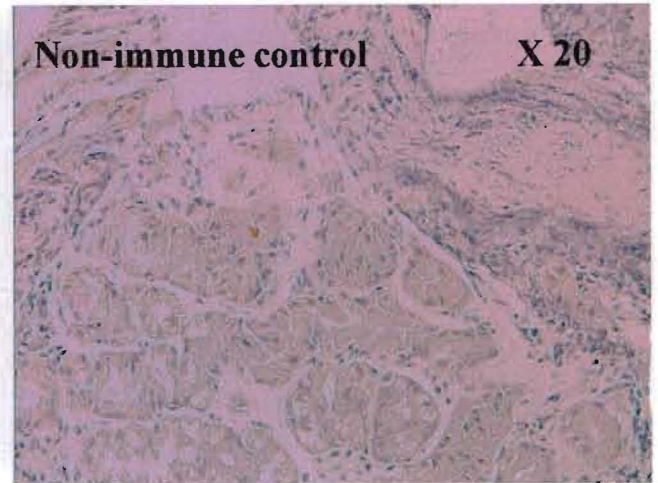
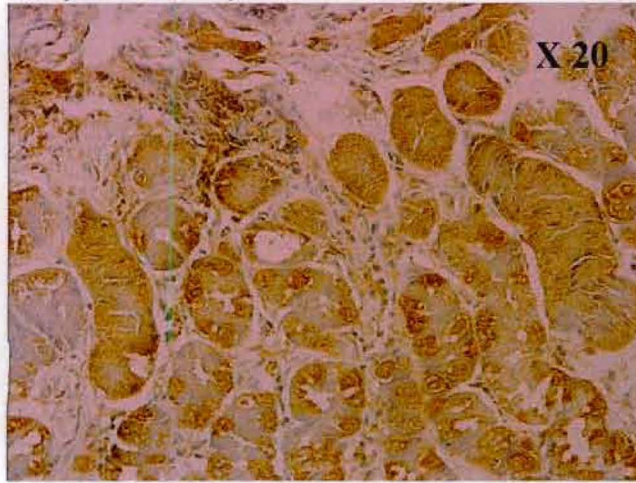


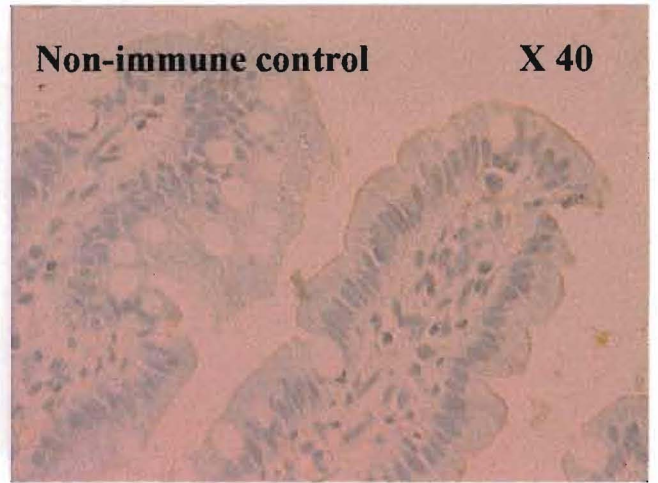
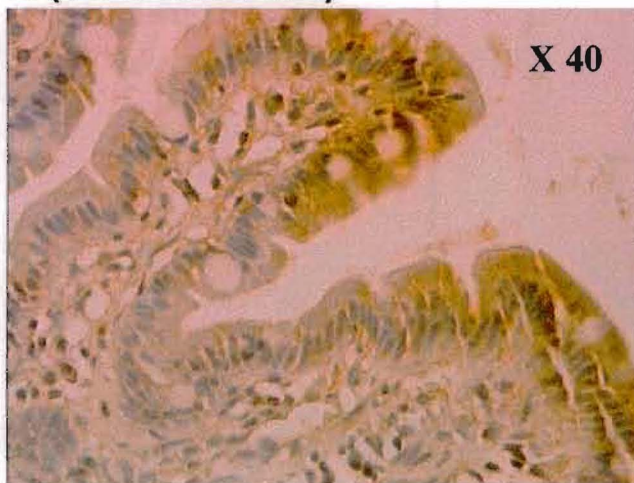
Figure 8.2 Selected stained sections (left column) and their corresponding non-immune controls (right column):

- A** strong PPO staining of the hepatocytes
- B** strong staining in the proximal convoluted tubules of the kidney
- C** strong staining in the serous cells of the peribronchial glands in the lung

D (Stomach)



E (Small intestine)



F (Large intestine)

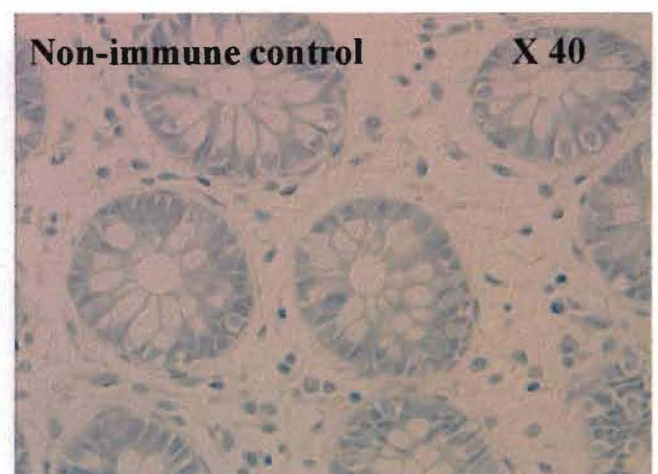
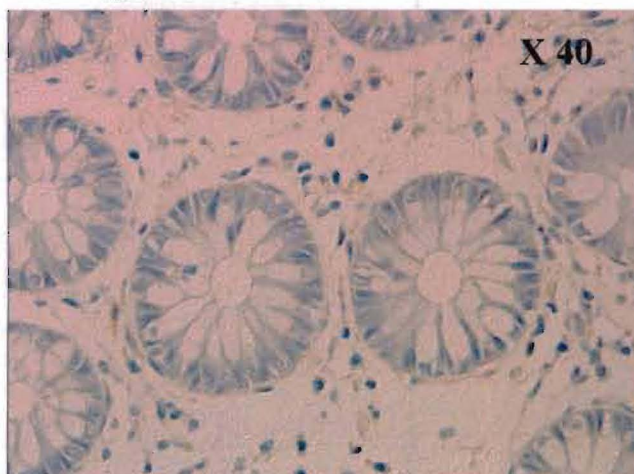
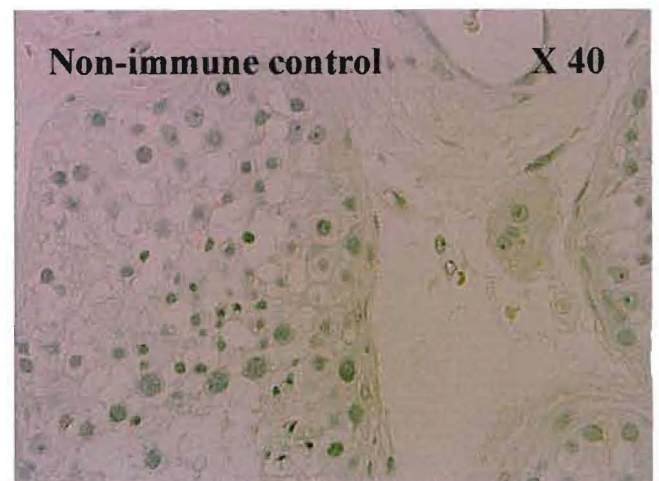
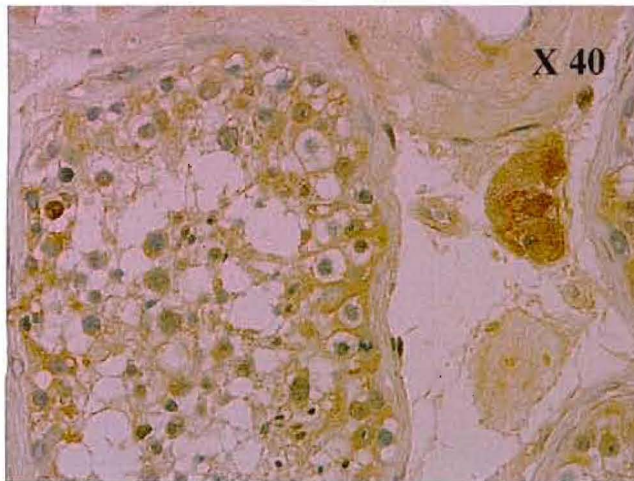


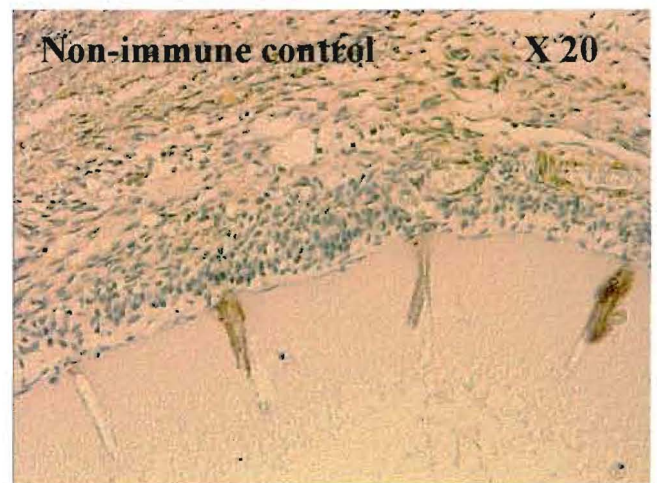
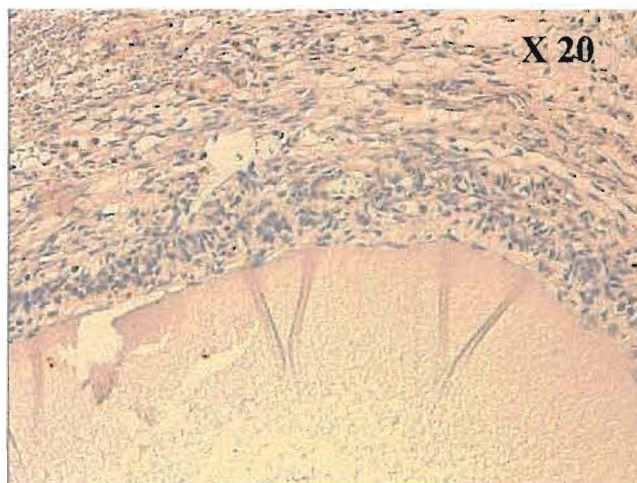
Figure 8.2 Continued:

- D strong PPO staining of the parietal cells in the stomach
- E PPO staining present in tips of villi in small intestine
- F PPO staining absent in large intestine

G (Testes)



H (Ovary)



I (Placenta)

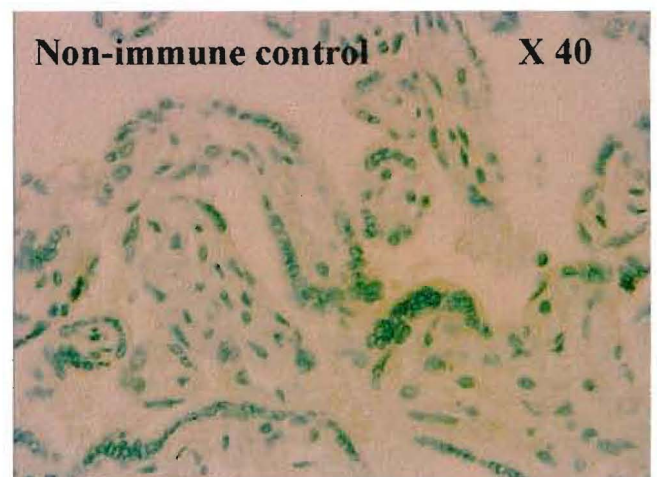
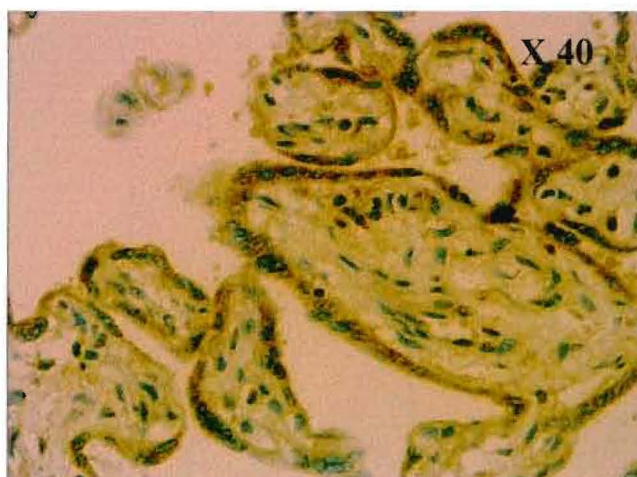


Figure 8.2 Continued:

- G** strong PPO staining of the interstitial cells in the testes
- H** negative PPO staining of a mature follicle in the ovary
- I** PPO staining of the cytotrophoblasts in the placenta

All 5 kidneys exhibited strong staining of the cytoplasm in the proximal convoluted tubules. There was moderate to strong staining in the collecting tubules. No staining for PPO was observed in the distal convoluted tubules or the glomeruli. All non-immune controls were negative (figure 8.2 B).

The bronchial epithelium and the alveolar macrophages showed moderate staining for PPO. In one case, peribronchial glands were present and showed negative staining in the mucous cells but strong positive staining in the serous cells (figure 8.2 C). All non-immune controls were negative. All the lung specimens contained carbon pigment.

In the stomachs there was strong cytoplasmic staining of parietal cells. No positive staining was noted in any of the non-immune controls (figure 8.2 D).

The staining in the intestine varied. PPO staining in all cases was strongest in the proximal small intestine and involved cells at the tips of the villi, but not cells at the base of the crypts which were unstained (figure 8.2 E). Mucosal epithelial staining became progressively weaker as more distal portions of the bowel were examined. Indeed, epithelial cell staining in the colon was weak to negative (figure 8.2 F). However, mucosal macrophages also showed moderate cytoplasmic staining for PPO. No staining of plasma cells anywhere in the gut was noted.

In all 5 testes the columnar cells of the seminiferous tubules stained moderately. When present, the interstitial (Leydig) cells, stained strongly (figure 8.2 G). In two sections the epithelium of the epididymus was present and showed moderate staining. Staining of spermatozoa was impossible to assess at this magnification. No staining was seen in the non-immune controls.

From five ovarian sections examined, primitive follicles were observed in three and no staining for PPO were observed in these cells. In four out of five sections, the mature follicular cells showed negative staining. However, in one case the epithelium of one mature follicle showed strong staining. All non-immune controls were negative (figure 8.2 H).

In all five placental tissue sections examined, the cytotrophoblasts showed a moderate staining of the cytoplasm for PPO (figure 8.2 I). All non-immune controls were negative. As all the placentas were from cases of full term pregnancies no syncytiotrophoblasts were present.

Electron microscopy - Subcellular distribution of PPO in human hepatocytes

Figure 8.3 is an electron micrograph showing transformed *E. coli* cells which express recombinant human PPO. As expected a high concentration of specifically labelled PPO, visualised as a mass of small black dots which represent the 5 nm gold particles attached to the bound PPO antibody, was seen in these cells. This could be compared

E.coli

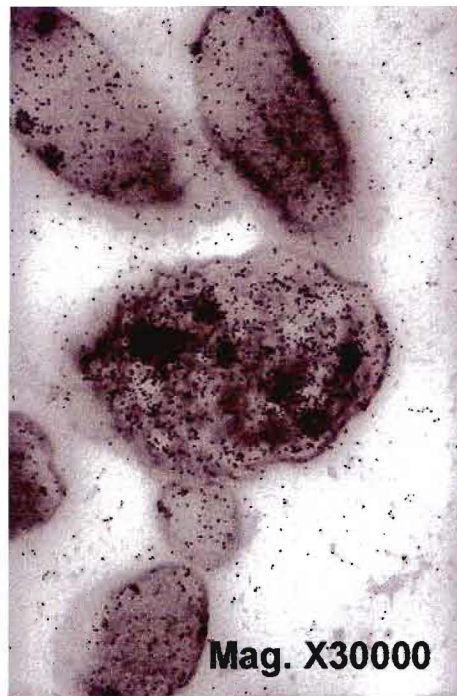


Figure 8.3 Electron micrograph of *E.coli* expressing-PPO showing positive immunogold labelling of PPO (left) when compared to non-immune control (right).

Liver

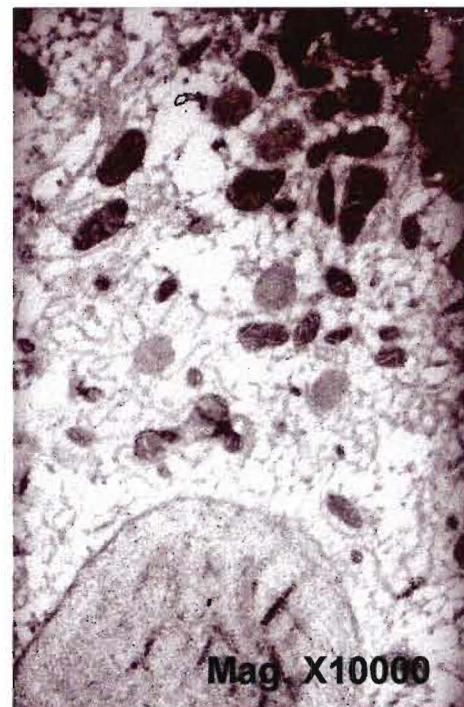
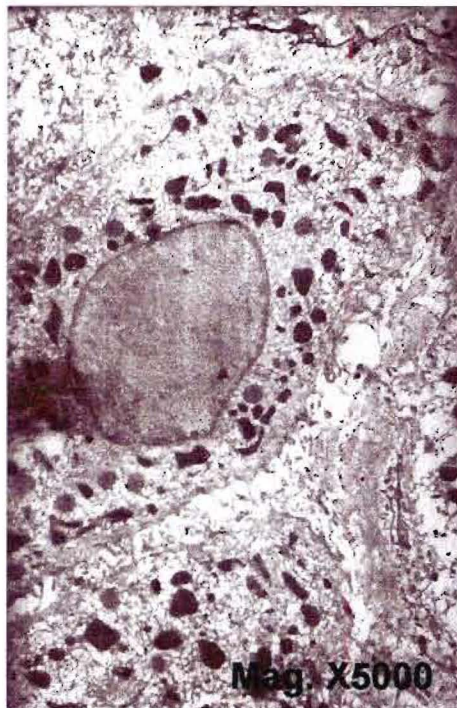


Figure 8.4 Electron micrograph (X5000, left; X10000, right) of liver biopsy specimen demonstrating ultrastructure preservation.

Liver mitochondria

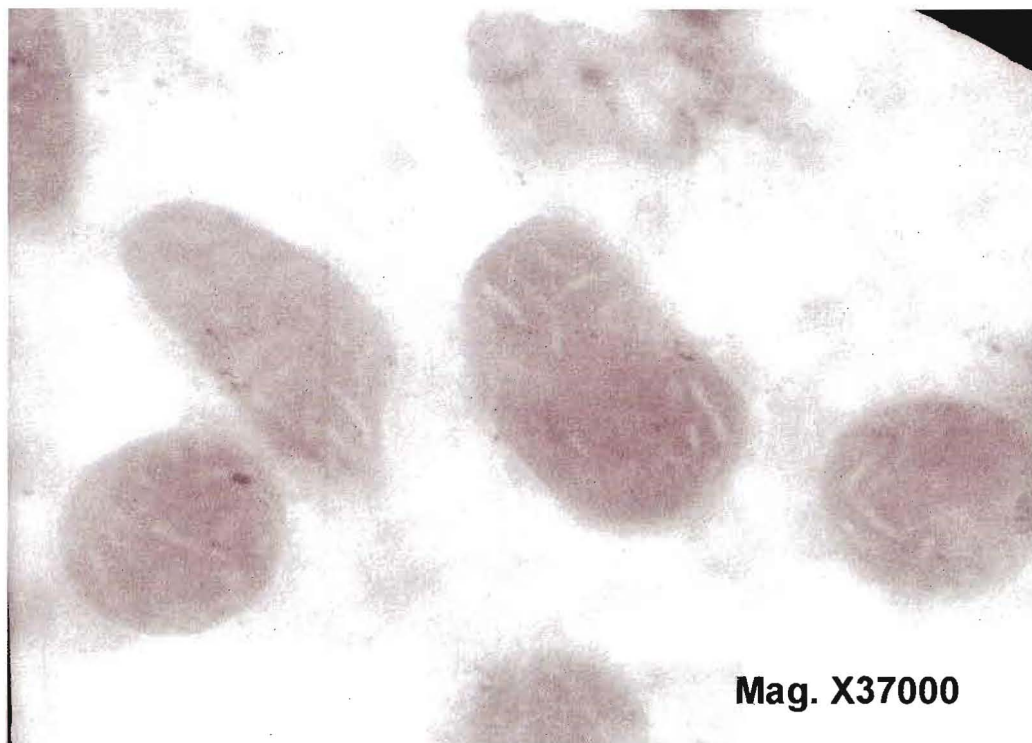
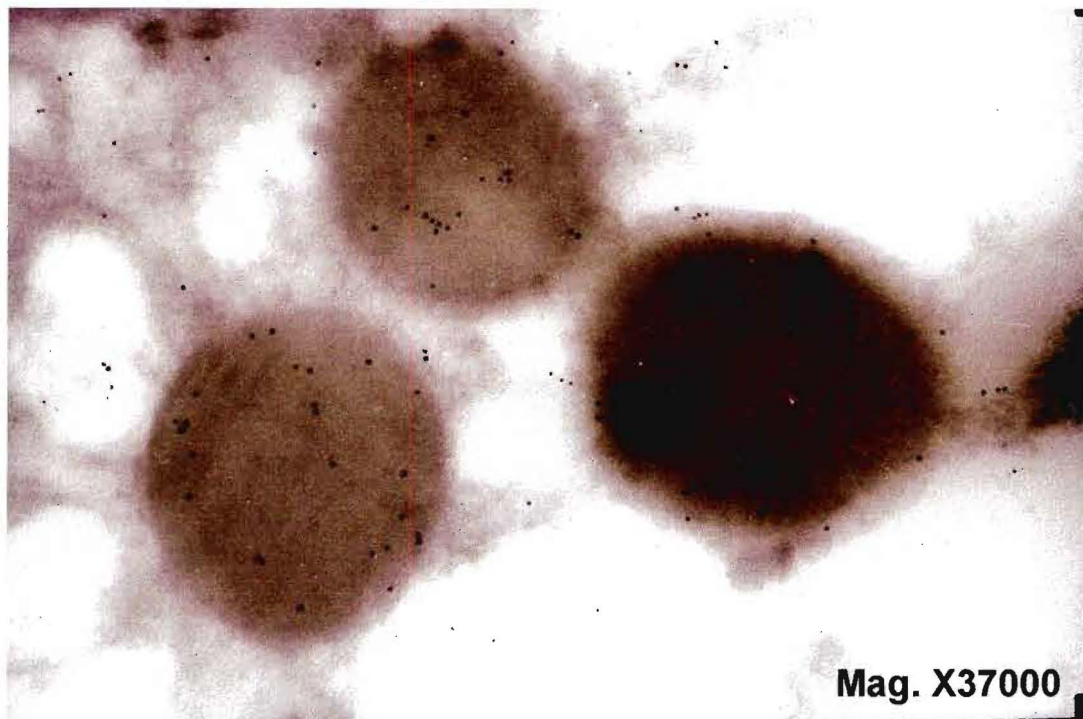


Figure 8.5 Electron micrograph of liver cell showing positive immunogold labelling of PPO in the mitochondria and some in the cytoplasm (top) when compared to non-immune control (bottom).

to the relative absence of labelling in equivalent non-immune controls, which had been incubated with normal rabbit serum.

The electron micrograph in figure 8.4 shows a section of a liver cell with a well preserved nucleus and several distinct mitochondria within the cytoplasm, thus demonstrating adequate preservation of tissue integrity.

Figure 8.5 shows mitochondria labelled with gold particles, demonstrating the presence of PPO in these organelles. Interestingly, there was lesser but significant labelling in the cytoplasm. Minimal or no gold particles were observed in the nuclei. This labelling pattern was observed in several experiments with liver tissue sections from different patients. All non-immune controls showed minimal labelling.

These observations were quantified by counting the number of gold particles per unit area (μm^2) and the results shown in table 8.1. This is represented graphically in figure 8.6. The number of gold particles per μm^2 area in sections labelled with PPO antibody ranged from 91 – 139 (mitochondria) and from 15 – 39 (cytosol), whilst in sections labelled with non-immune rabbit serum, the range was 0 – 10 (negative controls). The results represent data from 5 different livers.

Table 8.1 Immuno-gold labelling of liver tissue sections using the human PPO antibody.

Mitochondria+PPO-Ab (Gold particles/ μm^2)	Cytosol+PPO-Ab (Gold particles/ μm^2)	Negative Controls (Gold particles/ μm^2)
113 \pm 18 (n = 5)	24 \pm 3 (n = 5)	6 \pm 4 (n = 6)

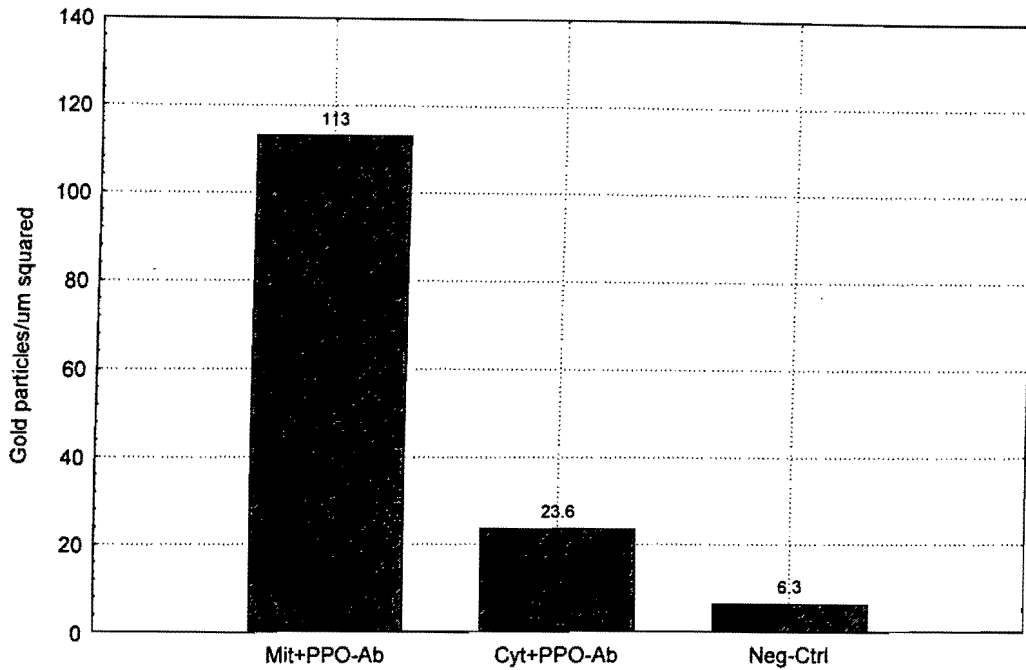


Figure 8.6 Immuno-gold labelling of human liver tissue sections with PPO antibody (PPO-Ab), and with normal rabbit serum used for negative controls (Neg-Ctrl), showing the predominant location of PPO within the mitochondria and its presence, to a lesser extent, in the cytosol. Mit = mitochondria, Cyt = cytosol.

In an attempt to further minimise non-specific staining IgG was extracted from the rabbit serum in which PPO antibody had been raised (see Appendix 10) by ammonium sulphate precipitation (Hebert et al, 1973). However, non-specific binding was not reduced.

Discussion

Generally, cellular activity and the demand for energy is reflected by the number of mitochondria in the cells. In highly active cells where there is a high haem requirement there will be large numbers of mitochondria, whilst in less active cells there will be few. As the mitochondrion is the predominant location of PPO, the number of mitochondria should correspond to the relative staining for PPO.

The liver is a multifunctional organ in the body involved in several metabolic activities such as synthesis of proteins, lipoproteins, conversion of sugars into glycogen for storage, degradation of various toxic products, and several other processes. To produce the energy required for these processes hepatocytes contain a large number of mitochondria, which are the predominant locations of PPO, hence these cells stain strongly for this protein. The phagocytic Kupffer cells are relatively less active with fewer mitochondria, hence the lack of staining for PPO.

In the kidneys the proximal convoluted tubules are lined by simple cuboidal epithelial cells with microvilli which contain large numbers of mitochondria. These are very active cells involved in the reabsorption of approximately 75% of ions and water from the renal tubules into blood capillaries. In addition to the active transport of Na^+ ions, these cells also engulf proteins from the tubules into endocytic vesicles which link up with lysosomes for their degradation. The distal convoluted tubules are relatively less active and possess fewer mitochondria hence their lack of staining for PPO. Similarly the glomeruli are less active, involved in the filtration of water and dissolved substances down a pressure gradient, and this process requires less energy production by the cells. It follows therefore that these cells will have much fewer mitochondria, and therefore will stain less or not at all for PPO.

The ciliated bronchial epithelium stains positively for PPO. These cells are involved in mucus production and the co-ordinated movement of mucus by a sweeping movement of the cilia along the respiratory tract, both of which are energy requiring processes. Similarly, the serous cells in bronchial glands which contain secretory granules, for the secretion of peptide compounds stain strongly. In contrast, the mucous cells which produce and store mucus contain fewer mitochondria hence, they lacked staining for PPO.

The parietal cells of the stomach are secretory cells which produce and secrete HCl and other factors. These cells contain large numbers of mitochondria which reflect the high energy need for production and secretion of these substances and thus a high haem requirement. The strong staining for PPO observed in these cells supports this.

Similarly, in the small intestine, the cells at the tips of the villi, which are involved in the absorption of nutrients and the synthesis of digestive enzymes stained strongly. These cells contain numerous mitochondria to provide the energy requirement for these activities. On the other hand, the cells at the base of the crypts of Lieberkuhn, did not stain for PPO. The crypts are composed mainly of stable cells with a low turnover.

In the testes, the stratified columnar cells lining the seminiferous tubules are a proliferating population involved in spermatogenesis. Between these cells are the secretory Leydig cells which produce and secrete the male hormone, testosterone. These are very active populations of cells, hence the strong staining for PPO. The primitive follicles in the ovary contain a population of dividing cells which are relatively inactive, hence they stain negatively for PPO. In mature follicles most of the cells are still dividing, but at a certain stage some of the cells will start producing and secreting female sex hormones, oestrogen and progesterone. Depending on the activity of the cells and therefore the energy requirement which is reflected by the number of mitochondria, these follicles may stain positively for PPO. This may explain the positive staining in one of the sections. Similarly, the cytotrophoblasts in the placenta, which contains relatively undifferentiated stem cells which produce peptide hormones, oestrogen and progesterone, will show features of energy demand. Thus there was cytoplasmic staining of these cells.

In initial electron microscopy experiments it was realised that the immediate transfer of the liver tissue into our prepared fixation solution was crucial to preservation of tissue ultrastructure. We had observed that tissue left in the formalin solution used for collecting samples for histopathological diagnosis at Groote Schuur hospital resulted in deterioration of ultrastructure. As the amount of liver tissue required for our purposes was very small it proved unnecessary to fix the tissue by puncture perfusion as is often described by others (Perroti et al, 1987). Our methodology enabled us to achieve adequate preservation of both tissue integrity and antigenicity of PPO. The staining procedure used also produced good contrast for the electron microscope visualisation of different cellular organelles.

In this study cryoultramicrotomy and immuno-gold labelling were utilised to study the subcellular localisation of PPO in human liver biopsies. PPO was shown to be predominantly located in the mitochondria of human hepatocytes as well as the cytoplasm though, to a lesser extent. The mitochondrial location of PPO was not unexpected as it has previously been reported in subcellular fractionation studies that PPO activity was associated with mitochondrial membranes (Poulson and Polglase, 1975; Poulson, 1975; Jacobs and Jacobs, 1981 and 1984; Deybach et al, 1985, Ferreira et al, 1988, Camadro et al, 1994). Previous subcellular fractionation studies on photosynthetic cells showed that, in addition to the mitochondria, PPOs were also associated with chloroplasts (Matringe et al, 1992b; Smith et al, 1993). Although no specific mitochondrial targeting signal has been identified in the human PPO sequence, it has been established that the mammalian protein, which is encoded by nuclear DNA (Kolarov et al, 1983), is imported into mitochondria. Import studies with recombinant PPOs from human (Nishimura et al, 1995b) and tobacco plant (Lermontova et al, 1997) showed that these proteins were targeted and translocated into these organelles. In plants, in addition to the mitochondria, PPO activity has been associated with other subcellular structures such as the plasma membrane (Jacobs et al, 1991; Lee et al, 1993) and the endoplasmic reticulum (Retzlaff and Boger, 1996).

The interesting visualisation of significant immunogold labelling within the cytoplasm, indicating presence of PPO in this cellular compartment may lend support to the suggestion of precursor forms of the protein which are presumably destined for the mitochondria. Previous studies showing little or no cytoplasmic PPO activity could indicate that these precursor forms of the protein have very little or no activity at all. The existence of precursor forms of PPO would be in keeping with the synthesis of other mitochondrial proteins as precursors, which facilitates their targeting and translocation into these organelles. Previous findings have indicated that synthesis of the mammalian (rat) PPO does not take place on mitochondrial, but rather on cytoplasmic ribosomes (Kolarov et al, 1983).

Concluding Remarks

The presence of PPO has been demonstrated in a wide variety of organs. It therefore appears that haem synthesis occurs in a variety of tissues, and generally this process correlates with a cell's demand for energy production. As haem is an important component of many proteins involved in energy transfer and redox reactions, it stands to reason, that there will be a variable requirement for haem that is tissue/cell dependent. This is reflected in the presence/absence of haem synthetic enzymes, of which PPO is representative. While our observations are largely descriptive, the results imply that there are some significant sites of haem synthesis other than the liver and bone marrow which should not be ignored in studies concerned with haem dynamics and flux in the human body.

Our electron microscopy studies demonstrated that human PPO is predominantly located in mitochondria in liver cells, confirming previous findings from sub-cellular fractionation studies. This work has also demonstrated the presence of immunologically detectable PPO in the cytosol, which may indicate the existence of precursor forms of this protein in this compartment.

The techniques developed in this study may prove to be a useful tool in understanding the pathogenesis of VP, particularly that due to PPO mutations which may disrupt the targeting and translocation of this protein into the mitochondria.

This study also underlines the possibility that immunocytochemical techniques in combination with electron microscopy may allow the study of precursor forms of proteins, other than PPO.

Chapter Nine

Conclusions and Future Directions

In this work interspecies characterisation of PPOs from human, *B. subtilis* and *M. xanthus* was carried out. The use of a rapid single-step purification procedure with the recombinant proteins has been a great improvement when compared to previous methods which involved multiple steps that often resulted in significant protein loss. The ability to produce and work with purified, active recombinant PPOs facilitated the characterisation of these enzymes.

Data from substrate specificity and lipid effects revealed differences and similarities in the characteristics of *B. subtilis*, *M. xanthus* and human PPOs. Similarly, data from the investigation of these PPOs by means of inhibitor studies highlighted the differences between *B. subtilis*, *M. xanthus* and human. Various inhibitor substances yielded a competitive mode of inhibition, probably due to electronic and structural similarities to the PPO substrate, protoporphyrinogen-IX. The behavioural differences between different forms of this protein were clearly not a simple reflection of prokaryotic versus eukaryotic diversification, nor Gram positivity versus negativity in the case of prokaryotes.

Primary structural variations clearly affect the ability of the protein to interact with different compounds, be it substrates or inhibitors. Investigation of the effects of changes in protein/gene sequences, arising from both natural and engineered mutations, was not the goal of this work but, substrate and inhibitor studies on the different forms of the protein may provide clues to amino acid residues involved in catalysis and/or substrate binding. This work has shown that such detailed kinetic studies are indeed feasible.

Phylogenetic analysis based on PPO protein sequences revealed interesting divergences, especially, amongst prokaryotic forms. Generally, grouping of the various organisms in the phylogenetic tree was similar to those obtained using other methods based on morphology and/or fossil records. This work shows the use of protein or gene sequences as highly suitable for establishing phylogenetic relationships because of the vast amount of information they contain.

Demonstration of PPO in human tissues/organs indicates occurrence of haem synthesis in a variety of tissues. It appears that PPO is more apparent in "active" cells where energy demand is greater. Localisation studies using electron microscopy confirmed the predominant location of PPO in mitochondria, and a cytosolic form, which probably represents precursor forms, was demonstrated by immunogold labelling. This methodology may provide yet another useful tool in investigating the pathogenesis of variegate porphyria, particularly due to PPO

mutations which may disrupt the targeting and translocation of these proteins to the mitochondria.

It is clear that the use of molecular biology techniques has facilitated the characterisation of various proteins, including the haem synthetic enzymes. This has enabled refinement of purification procedures resulting in simple rapid steps with large yields of native proteins. Detailed characterisations are therefore possible, and in some cases protein crystal structures have been determined. As more detailed data, such as is presented in this dissertation, becomes available, it will be possible to identify and isolate critical elements of PPO with respect to catalysis and structure. Importantly, data emanating from the application of these techniques to both naturally occurring and engineered mutant PPOs will assist in the elucidation and further understanding of the pathogenesis of VP. Once this is understood, rational approaches to long-term therapy (or conceivably cure) may be possible.

APPENDICES

EXPERIMENTAL METHODS AND MATERIALS

Appendix One

Transformation, Expression and Purification of the Recombinant Protoporphyrinogen Oxidases of *B. subtilis*, *M. xanthus* and Human

1.1 Transformation of *E. coli* JM 109 cells by PPO expressing recombinant plasmids

Reagents and equipment

1. Luria-Bertani (LB) medium

10 g Tryptone (DIFCO Laboratories, Detroit, Michigan, USA)

5 g Yeast Extract (DIFCO Laboratories, Detroit, Michigan, USA)

5 g NaCl

made up to 1 L with deionised water and autoclaved.

For antibiotic resistance selective media 100 µg/ml ampicillin was added to the broth, once cooled to approximately 40°C, and mixed.

2. LB agar plates with ampicillin (100 µg/ml)

2 g Tryptone

1 g Yeast Extract

1 g NaCl

3 g Agar Noble (Difco Laboratories, Detroit, Michigan, USA)

made up to 200 ml with deionised water and autoclaved. Media was cooled to 40°C, ampicillin (100 µg/ml) added, and quickly poured into sterile petri plates under a sterile hood before agar solidified.

3. Calcium Chloride solution (pH 8.0)

-50 mM CaCl_2

-10 mM Tris/HCl

-sterilized by autoclaving.

4. Sterile 1.5 ml microfuge tubes

5. Heating block (Techne dri-block DB-2D, Laboratory and Scientific equipment Co. Cape Town, South Africa).

6. Ice bath.

7. Recombinant plasmid vector (pBsubtPPO-X, pHPPPO-X or pMx-PPO).

Transformation procedure

-1 ml of an overnight culture (10 ml) of *E. coli* JM 109 cells was transferred into 100 ml of sterile LB broth in a 500 ml flask. The cells were grown with vigorous shaking at 37°C until an O.D.₆₀₀ of 0.5 was achieved.

-The culture was decanted into pre-chilled sterile 50 ml capped tubes and chilled on ice for 10 min. The cell suspension was centrifuged at 4 000 G for 5 min at 4°C.

-The cell pellet was resuspended in ice-cold sterile CaCl_2 solution (pH 8.0), which was half the original volume of medium used for the culture. This was placed in an ice bath for 15 min and then centrifuged at 4 000 G for 5 min at 4°C.

-The cells were resuspended in ice-cold sterile CaCl_2 solution (pH 8.0), which was 1/15 the original volume of medium used for the culture. 200 µl aliquots were dispensed into pre-chilled sterile Eppendorf tubes. These were stored at 4°C for 12-24 h to increase competence.

-Recombinant plasmid DNA (approximately 40 ng in a maximum 100 µl TE buffer) was added to an aliquot of the cell suspension, mixed and stored on ice for 30 min.

-The transformation mixture was transferred to a heating block preheated to 42°C, and left for 2 min.

-1 ml of sterile LB broth was added, and incubated at 37°C for 1 h without shaking, to allow the bacteria to recover and begin expressing antibiotic resistance.

-100 μ l, 200 μ l, and 500 μ l of transformed cell suspension was spread out with a sterile glass spreader over LB plates containing ampicillin (100 μ g/ml).

- Once the cell suspension had absorbed into the medium the plates were incubated at 37°C for 12-16 h until colonies appeared. Apparent single colonies were then replated on fresh plates. After appearance of single colonies, 100 ml of LB broth containing ampicillin (100 μ g/ml) was inoculated with a single colony.

-After overnight growth 700 μ l aliquots of the culture were transferred into microfuge tubes with 300 μ l sterile glycerol, mixed and stored at -80°C as stocks.

1.2 Expression and Purification

Reagents and equipment

-Orbital shaker incubator (Yih der LM-510, Taiwan)

-LB medium with 100 μ g/ml ampicillin.

-Ni-NTA agarose (Qiagen, Chatsworth CA, USA).

-Talon resin (Clontech Laboratories, Palo Alto, USA).

-Sonicator Ultrasonic Processor XL (Heat Systems Inc, Farmingdale, New York, USA).

-Phenyl-methyl-sulfonyl fluoride (PMSF), 1 μ g/ml final concentration.

-Isopropyl-1-thio- β -D-galactoside (IPTG); 1 M stock.

-Jouan KR 422 centrifuge (Saint Herblain, France).

-Beckman L7-65 Ultracentrifuge (Beckman Instruments, Inc., Palo Alto, California, USA).

1.2.1 Expression and purification of the *B. subtilis* PPO

1.2.1.1 PPO expression

-100 µl of thawed *E. coli* cells containing the pBsubtPPO-X plasmid, which expresses recombinant *B. subtilis* PPO, was transferred into 10 ml of sterile LB medium containing ampicillin (100 µg/ml) and incubated overnight at 37°C.

-1 ml of the culture was transferred into 1 L of LB medium (in a 2 L flask) and incubated with vigorous shaking at 37°C until an O.D.₆₀₀ of 0.5-0.6 was reached. IPTG (1 mM) was added to induce PPO expression and cell growth continued for another 4 h.

-Cells were harvested by centrifugation at 4 000 G for 30 min at 4°C.

1.2.1.2 Native purification of recombinant *B. subtilis* PPO:

-The cell pellet was resuspended in 30 ml of ice-cold sonication buffer (50 mM NaH₂PO₄, 300 mM NaCl, 1% Tween-20, pH 8.0).

-The cell suspension was sonicated 4 X30 sec on ice to produce a lysate. This was ultracentrifuged at 40 000 G for 30 min at 4°C.

-Approximately 30 ml of PPO-containing supernatant was loaded onto a Ni-NTA agarose column (0.9 X 1 cm), pre-equilibrated with sonication buffer (15 ml), and allowed to flow through by gravity.

-The column was washed with sonication buffer (20 ml), followed by 20 ml of the first wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 0.2% Tween-20, pH 8.0) and 20 ml of a second wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 0.2% Tween-20, 10% glycerol, pH 6.3)

-Bound PPO was eluted from the column with 100 mM imidazole in the second wash buffer.

-PMSF (1 µg/ml) was added, immediately before use, throughout the purification procedure.

1.2.1.3 Purification of *B. subtilis* PPO using denaturing conditions

-The cell pellet was resuspended in denaturing solubilisation buffer (6 M GuHCl, 100 mM Na-phosphate, 10 mM Tris, pH 8.0) at 5 ml per gram wet weight and stirred gently for 1 h at room temperature.

-The lysate was centrifuged at 105 000 G for 30 min at 4°C and the PPO-containing supernatant was collected.

- The supernatant was loaded onto a Ni-NTA column (0.9 X 1 cm) pre-equilibrated with the solubilisation buffer at a rate of 3 column vol/h.
- The column was then washed with solubilisation buffer (15 ml), followed by 20 ml of a first wash buffer (8 M urea, 100 mM Na-phosphate, 10 mM Tris, pH 8.0) and 15 ml of a second wash buffer (8 M urea, 100 mM Na-phosphate, 10 mM Tris, pH 6.3).
- Bound protein was eluted from the column with 250 mM imidazole in the second wash buffer.
- PMSF (1 µg/ml) was added immediately before use throughout the purification procedure.

1.2.2 Expression and purification of *M. xanthus* PPO

1.2.2.1 PPO expression

- 1 ml from an overnight culture (10 ml) of *E. coli* cells, containing the pMx-PPO plasmid which expresses recombinant *M. xanthus* PPO (Dailey and Dailey, 1996a), was transferred into 1L of LB medium with ampicillin (100 µg/ml) and incubated at 37°C with vigorous shaking for 20 h.
- Cells were harvested by centrifugation at 105 000 G for 30 min at 4°C.

1.2.2.2 PPO purification by Talon affinity chromatography using native conditions

- The cell pellet was resuspended in 30 ml of sonication buffer (20 mM Tris/HCl, 100mM NaCl, 1% Tween 20, pH 8.0).
- The cell suspension was sonicated 4 X 30 sec, and the resulting lysate centrifuged at 105 000 G for 30 min at 4°C to produce a PPO-containing supernatant.
- The supernatant was loaded onto a 1 cm Talon resin column pre-equilibrated with sonication buffer (15 ml) and allowed to flow through by gravity.
- The column was washed with 15 ml of sonication buffer, followed by 20 ml of a first wash buffer (20 mM Tris/HCl, 100 mM NaCl, 10% glycerol, 0.5% Tween-20, pH 6.3), 20 ml of a second wash buffer (50 mM NaH₂PO₄, 100 mM NaCl, 0.5% Tween-20, 10% glycerol, pH 6.3) and finally 20 ml wash buffer 2 containing 25 mM imidazole, pH 6.3.
- Bound protein was eluted with 100 mM imidazole in the second wash buffer.
- PMSF (1 µg/ml) was added immediately before use throughout the purification procedure.

1.2.3 Expression and purification of human PPO

1.2.3.1 PPO expression

-1 ml of an overnight culture of *E. coli* cells containing the pHPPO-X plasmid, from which recombinant human PPO is expressed (Dailey and Dailey, 1996b), was inoculated into 1 L of sterile LB medium with ampicillin (100 µg/ml).

-The cell culture was incubated at 37°C for 22 h with vigorous shaking after which the cells were harvested by centrifugation at 4 000 G for 30 min at 4°C.

1.2.3.2 Purification of human PPO by Talon affinity chromatography using native conditions

-The cells were resuspended in 30 ml of sonication buffer (20 mM Tris/HCl, 300 mM NaCl, 1% n-octyl-β-D-glucopyranoside, pH 8.0) and subjected to 4 X 30 sec sonication on ice.

-The lysate was centrifuged at 105 000 G for 30 min at 4°C and the PPO-containing supernatant collected.

-The supernatant was loaded onto a Talon resin column (approximately 1 cm) pre-equilibrated with sonication buffer (15 ml) by gravity.

-The column was washed with sonication buffer (20 ml) followed by 20 ml of wash buffer (20 mM Tris/HCl, 300 mM NaCl, 25 mM imidazole, 0.2% n-octyl-β-D-glucopyranoside, pH 8.0).

-Bound protein was eluted from the column in 20 mM Tris/HCl, 300 mM NaCl, 0.2% n-octyl-β-D-glucopyranoside 150 mM imidazole, pH 8.0.

-PMSF (1 µg/ml) was added immediately before use throughout the purification procedure.

Appendix Two

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis [SDS-PAGE]

(Laemmli, 1970)

Reagents and equipment

1. SE-600 vertical slab gel electrophoresis unit (Hoefer Scientific Instruments/Pharmacia Biotech, Cambridge, UK)
2. PS-1200 DC power pack (Hoefer Scientific Instruments/Pharmacia Biotech, Cambridge, UK)
3. 30 ml gradient mixer (Hoefer Scientific Instruments/Pharmacia Biotech, Cambridge, UK)

Electrophoresis gel

A 3.6% stacking gel in 125 mM Tris/HCl, pH 6.8 on a 7.5%-17.5% linear gradient acrylamide gel in 350 mM Tris/HCl, pH 8.8 was used for the separation of proteins by electrophoresis.

17.5% Resolving gel solution

- 7 ml of "high" buffer (1 M Tris/HCl, pH 8.8; 30% Glycerol).
- 12 ml of A-Bis-A solution (30% acrylamide/0.8% bisacrylamide).
- 0.3 ml of 10% SDS.
- made up to 20 ml with deionised water.

7.5% Resolving gel solution

- 7 ml of "low" buffer (1M Tris/HCl, pH 8.8; 7.5% Glycerol)
- 5 ml of A-Bis-A solution (30% acrylamide/0.8% bisacrylamide).
- 0.3 ml of 10% SDS
- made up to 20 ml with deionised water.

Spacer solution for stacking gel

- 8.6 ml Spacer buffer (125 mM Tris/HCl, pH 6.8)
- 1.2 ml A-Bis-A solution (30% acrylamide/0.8% bisacrylamide).
- 0.1 ml 10% SDS

Gel electrophoresis tank buffer

- 25 mM Tris, pH 8.8
- 200 mM Glycine
- 0.1% SDS

PAGE-83 stain

- 1 g PAGE-83
- 200 ml 50% TCA
- 300 ml Ethanol
- 500 ml deionised water

Filtered before use.

SDS sample loading solution

(Maizel, 1971)

- 20% Glycerol
- 2%SDS
- 0.002% Bromophenol Blue
- 0.2% β -mercaptoethanol
- in 0.005 M Tris/HCl pH 6.8.

Gel destain solution

- 320 ml Glacial acetic acid
- 1L ethanol
- made up to 4L in deionised water.

Preparation of SDS Polyacrylamide gel

- 20 ml each of the 17.5% and 7.5% resolving gel solutions were prepared.
- To each resolving gel solution 100 μ l of freshly prepared 5% ammonium persulphate solution and 10 μ l of TEMED were added immediately before use, and mixed well.
- 16 ml of the 7.5% resolving solution was poured into the left chamber of the gradient mixer allowing a small volume to flow into the channel connecting the right chamber which was positioned on top of a magnetic stirrer.
- 16 ml of the 17.5% resolving solution was poured into the right chamber which contained a rotating stirrer bar.
- Both the connecting tap and the tap leading from the gradient mixer were opened, and the mixing solution pumped from the gradient mixer to the gel well between the two glass plates mounted vertically on a gel pouring stand). The gel well was filled up to a level allowing the spacer comb teeth to be entirely within the stacking gel.
- Approximately 1 ml of water was gently layered above the gel using a syringe.
- Once set, the water was removed. 100 μ l of 15% ammonium persulphate (freshly prepared) and 10 μ l of TEMED were added to the spacer solution, mixed and poured on top of the gel. Sample bay comb was inserted and the gel allowed to set.

Protein preparation and gel electrophoresis

- Protein samples were boiled for 5 min with an equal volume of sample solution.
- The chemically denatured protein and samples containing imidazole were mixed with equal volumes of loading buffer and incubated at 37°C in a water bath for 15 min prior to loading onto gel.
- A buffer tank was assembled with the gel plate component containing the set gel.
- The tank was filled with gel electrophoresis buffer.
- The samples (maximum 100 μ l) were loaded into the spacer bays with a Hamilton microsyringe.
- The gel was run at a constant voltage of 65 V for 16 h or until tracker dye had advanced to approximately 1 cm from bottom of gel.

- The gel was removed from the plates and stained with gentle shaking for approximately 1 h.
- The gel was transferred to destain solution and agitated until protein bands were clearly visible and the background well reduced.
- The gel was visualised on a light box and photographed.

Appendix Three

Molecular Weight Determination of Native *B. subtilis* PPO

Reagents and equipment

Isco fraction collector with UV monitor (Anatach Instruments, Johannesburg, South Africa).

Aldolase bovine serum albumin, ovalbumin and cytochrome C from Gel filtration calibration kits, Sephadex G200 (Pharmacia Biotech, Vienna, Austria)

Packing and running of column

6 g Sephadex G-200 was swollen in deionised water at 90°C for 5 h., allowed to cool and the gel degassed.

The gel was packed into a 100 X 1.4 cm column. The column was equilibrated with 10 mM phosphate pH 7.4, 100 mM NaCl, 0.2% n-octyl -D-glucopyranoside at a fixed flow rate of 20 ml/h.

2 mg of each standard was dissolved in 2 ml of the equilibration buffer and loaded onto the G-200 at 20 ml/h and identical sized fractions collected.

A standard curve was constructed by plotting the molecular weight versus the fraction number. This was repeated to ensure reproducibility.

A run was performed in which non-denatured *B. subtilis* PPO was included (final volume 2 ml loaded at a flow rate of 20 ml/h).

Appendix Four

Protein Estimation with Bicinchoninic Acid

Sigma Procedure No. TPRO-562 (Kit No. BCA-1), Sigma Chemical Co., St. Louis, USA.

Reagents and equipment

Hitachi U-1100 Spectrophotometer (Hitachi Koki Co. Ltd, Tokyo, Japan).

Water bath (37⁰C).

Bicinchoninic Acid Solution; B-9643 - Reagent-A.

Copper (II) Sulphate Pentahydrate 4% Solution; C-2284 - Reagent-B.

Protein Standard Solution- 1.0 mg/ml bovine serum albumin (BSA).

Assay procedure

-The protein determination reagent was prepared by adding 1 part Copper (II) Sulphate Pentahydrate 4% Solution (1.5 ml) to 50 parts Bicinchoninic Acid Solution (75 ml).

-A standard curve was constructed as shown below to enable determination of concentration of the unknown protein. Tubes were prepared in duplicate as follows:

BSA (μl)	Water (μl)
0	100
20	80
40	60
60	40
80	20
100	0
Unknown sample (μl)	
100	0
50	50

-2 ml of protein determination reagent were added to each tube. The tubes were then vortexed and incubated at 37°C for 30 min.

-Tubes were cooled to room temperature and the absorbance measured at 562 nm.

-Blanks of the buffer utilized (100 and 50 μl) were also included and a water blank was used to zero the machine.

-Absorbance of both the standards and the unknown sample were read at 562 nm. An appropriate dilution of the unknown sample was prepared to ensure that its absorbance was within the range of standards.

-A standard curve was prepared by plotting the known added BSA concentration (μg) versus the net absorbance at 562 nm. This standard curve was used to determine the amount of unknown protein.

Calculation of protein concentration (mg/ml) in unknown samples:-

(mg unknown protein/assay) / (ml unknown/assay) X dilution factor

Appendix Five

Determination of Protein Molecular Weights

Procedure

-Unknown molecular weights for the PPO proteins were determined by comparison with protein standards of known molecular size (Da) Rainbow™ coloured protein molecular weight markers (Amersham International plc, Amersham Place, Little Chalfont), viz. Myosin (220 000); Phosphorylase b (97 400); Bovine serum albumin (66 000); Ovalbumin (46 000); Carbonic anhydrase (30 000); Trypsin inhibitor (21 500); and Lysozyme (14 300).

-For each protein standard the relative migration values (R_f) was determined using the formula:-

Distance protein has migrated from origin/ Distance from origin to reference point

-Using the top of the resolving gel as the origin and the furthest travelled distance by any of the protein standards was used as the reference point.

-The logs of the protein standard molecular sizes were plotted against their respective R_f values to construct a standard curve which was used to determine the unknown molecular weights of the protein (PPO) samples. This was done by using the measured R_f value for interpolation from the graph.

Appendix Six

Determination of Isoelectric Point (Flat bed Isoelectric Focusing)

Reagents and equipment

- ISOBOX model HE950 (Hoefer Scientific Instruments/Pharmacia Biotech, Cambridge, UK).
- LKB 2197 power supply (LKB/Pharmacia Biotech, Cambridge, UK).
- Ampholine PAG plates (pH range 3.5-9.5); LKB/Pharmacia Biotech, Cambridge, UK).
- Anode electrolyte solution (100 mM H_3PO_4 in double distilled water).
- Cathode electrode solution (100 mM NaOH in double distilled water).

Sample preparation

- The *B. subtilis* PPO was purified under non-denaturing conditions by the method described in "Expression and Purification of PPOs". Protein samples (0.5 mg/ml) were desalted in pure water by gel filtration on a PD 10 column prepacked with Sephadex G-25.

Isoelectric focusing (pH range 3.5-9.5)

The isoelectric points (pI) for the unknown proteins were determined by comparison with standard proteins of known pIs. The Pharmacia Broad pI Calibration kit containing the following standard pI markers was used:

Protein marker	PI (24 +/-1.5 °C)
Amyloglucosidase	3.50
Methyl red dye	3.75
Soybean trypsin inhibitor	4.55
β-lactoglobulin A	5.20
Bovine carbonic anhydrase B	5.85
Human carbonic anhydrase B	6.55
Horse myoglobin acidic band	6.85
Horse myoglobin acidic band	7.35
Lentil lectin acidic band	8.15
Lentil lectin middle band	8.45
Lentil lectin basic band	8.65
Trypsinogen	9.30

-The PAG plate was slowly lowered onto sufficient liquid paraffin on the cooling plate to ensure a complete layer under the gel. The isobox was then cooled to 10°C for 20 min.

-Electrode strips, cut to the correct size, were soaked in the appropriate electrode solutions and applied to the long edges of the gel.

-Sample application wicks were positioned in a row, 5 mm apart on the PAGplate approximately 20 mm from the cathode.

-The electrodes were positioned onto the electrode strips and the electrofocusing lid placed in position.

-The gel was prefocussed for 10 min using the following conditions:-

Power (W)	Voltage (V)	Current (mA)	Time (min)
30	1 500	50	10

-15 μ l of the unknown protein (PPO) and the Broad pI Calibration Kit markers (pH 3-10) (reconstituted in 100 μ l double distilled water) were applied to separate sample wicks.

-The gel was focussed as shown below :-

Power (W)	Voltage (V)	Current (mA)	Time (min)
30	1 500	50	90

-The application wicks were removed 30 min prior to end of focusing.

Staining of proteins with Coomassie blue.

-The gel was incubated for 1 h in fixing solution (11.5% TCA; 3.4% Sulphosalicylic acid) with gentle shaking.

-Washed in destaining solution (25% Ethanol; 8% Acetic acid) for 5 min.

-Stained for 2 h in staining solution (0.12% Coomassie Brilliant Blue R-250 in destaining solution) with gentle shaking.

-Destained by several washes in destaining solution until background had cleared.

pI determination

The known pIs of the different markers (Y-axis) were plotted against the respective migration distances (X-axis) in the gel from the cathode edge of the gel, and draw a calibration curve (pH gradient profile) by connecting the points with straight lines. The unknown pIs of the sample proteins were determined from the pH gradient profile curve by interpolation, using their migration distances from the cathode edge of the gel.

Appendix Seven

Flavin Cofactor Analysis

(Koziol, 1971; Faeder and Siegel, 1973; Cerletti and Giordano, 1971)

Reagents and equipment

- FMN and FAD; 97% pure standards from Sigma Chemical Co., St. Louis, USA.
- Flavin extract preparation.
- 20% Trichloroacetic acid (TCA) (chilled to 4°C).
- 100 mM Na₂HPO₄/NaH₂PO₄ buffer, 0.1 mM EDTA, pH 7.4.
- 200 mM Na-acetate buffer, pH 3.5.
- 3 M Tris chilled to 4°C.
- Hitachi U-3200 Recording Spectrophotometer (Hitachi, Koki Co. Ltd, Tokyo, Japan).
- Hitachi 650-10S Fluorescence Spectrophotometer (Hitachi, Koki Co. Ltd, Tokyo, Japan)
- Beckman L7-65 Ultracentrifuge, Ti 50 rotor (Beckman Instruments, Inc., Palo Alto, California, USA).

Extraction and determination of PPO flavins

- Protein samples were mixed with equal volumes of cold 20% TCA and placed on ice for 15 min.
- The mixture was centrifuged at 105 000 G for 20 min. at 4°C .
- pH of the flavin-containing supernatant was immediately adjusted to 7.4 using 3 M Tris.
- Appropriate dilutions of known flavin standards (FAD and FMN), and the flavin containing supernatant were prepared with the phosphate buffer pH 7.4. (for neutral pH readings), and with acetate buffer pH 3.5 (for acidic pH readings).
- The absorption spectra of the neutral (pH 7.4) solutions were recorded from 600 to 280 nm ($\lambda_{\text{exc.450 nm}}$).

-The maximum fluorescence emission spectra were recorded from 600 to 480 nm ($\lambda_{\text{exc. 450 nm}}$) for both the neutral (pH 7.4) and acidic (pH 3.5) flavin dilutions.

Appendix Eight

Protoporphyrinogen Oxidase Assay

(Meissner et al, 1986)

Reagents and equipment

1. Cuvette Buffer:-

-100 mM Tris/HCl

-1 mM EDTA

-3 mM Dithiothreitol (DTT) - added immediately before use.

-pH 8.1 for human and *M. xanthus* PPOs, and 8.7 for *B. subtilis* PPO.

2. Assay Buffer:-

-same as cuvette buffer plus 0.1% Tween-20.

3. Substrates:-

-Protoporphyrin-IX (proto.) (Porphyrin Products, Logan, UT. USA).

-Mesoporphyrin-IX (meso.) and Coproporphyrin-III (copro.) (Porphyrin Products, Logan, UT, USA) were substituted for Protoporphyrin-IX when required.

4. 2.7 N HCl for dissolving Protoporphyrin-IX and 0.1 N HCl for Mesoporphyrinogen-IX and Coproporphyrinogen-III.

5. 100 μ M FAD solution.

6. 20% Ethanol in 10 mM KOH.

7. Sodium (Na) and Mercury (Hg) to prepare the 4% Na-Hg Amalgam for reduction of Protoporphyrin-IX.

8. Round botttomed glass flask with side arm.

9. Water bath (37⁰C) Memmert, Laboratory and Scientific, Cape Town, South Africa

10. Hitachi 650-10S Fluorescence Spectrophotometer.(Koki Co Ltd, Tokyo, Japan.
- 11.Hitachi U-1100 UV/VIS Spectrophotometer (Koki Co Ltd, Tokyo, Japan).
12. Bunsen burner.
13. pH meter
14. 2 M MOPS

The procedure was performed in a dark room.

Preparing protoporphyrin-IX stock solution

-12 mg protoporphyrin-IX was dissolved in 30 ml 10 mM KOH in 20% ethanol (v/v),with stirring in the dark (proto. stock). The solution was then filtered through a 0.45 µm filter.

-Protoporphyrin-IX concentration was determined by measuring absorbance of a 1/100 dilution of the proto in 2.7 N HCl at 408 nm in a u.v/vis spectrophotometer, using the extinction coefficient, 262.

$$[\text{Protoporphyrin-IX}] \mu\text{M} = \text{O.D.}_{408} / 262 \times 10^5$$

-The filtered proto. stock was diluted to a final volume of 12 ml in 10 mM KOH (230-250 µM final concentration). The exact proto. concentration was determined as described above and used to calibrate the fluorimeter.

Preparation of reaction and absorbance measurement (time point) tubes

-Assay reaction tubes: -enzyme sample (vol. as required) + FAD (50 µl) + assay buffer (to give final vol. of 1 ml), were prepared.

-For each assay tube a corresponding blank was prepared by substituting the enzyme sample with equal amount of heated protein (80°C for 10 min).

-Samples were preincubated in assay tubes for 20 min.

“Cuvette tubes”:- 1 ml cuvette buffer;1 tube per time point.

-Calibration of fluorimeter

- 1/10 dilution of proto. stock (100 μ l) was made in assay buffer (900 μ l) followed by a further 1/250 dilution (250 μ l) in cuvette buffer (6 ml). A standard curve was prepared as follows:-

1/250 dil. stock (μ l)	Cuvette buffer (μ l)
200	800
400	600
600	400
800	200
1000	0

-The fluorimeter was then calibrated such that the highest proto.concentration read approximately 1000 relative fluorescence units (RFU).

Preparation of substrate**1. Preparing Na-Hg amalgam (4%) for reduction of protoporphyrin-IX**

-1.8 g Na was cut into small pieces and covered with liquid paraffin; 43.2 g Hg weighed and both immediately put in a fumehood.

-The Hg was poured into the round bottom glass flask.

-Nitrogen gas was introduced through the angled side arm of the flask.

-The flask was held with a clamp and heated inside the fumehood.

-The Na was added piece by piece through the top of the vessel. The reaction may occur spontaneously, or require some gentle heating over the burner.

-The vessel was swirled around while heating, till amalgam was completely liquid and glass sides clear.

-Amalgam was immediately transferred into a pre-warmed mortar and agitated whilst chopping with a spatula until solidified. Agitation causes amalgam to condense into little granules.

2. Reduction of Protoporphyrin-IX to Protoporphyrinogen-IX with 4% Na-Hg amalgam

-Approximately half the amalgam was added to the round bottomed glass flask containing the diluted proto. The stoppered flask was shaken vigorously.

-Fluorescence was monitored under a uv light, and gas allowed to escape from time to time.

-As fluorescence disappeared the remaining amalgam was added and shaking continued until reduction was complete (as indicated by disappearance of fluorescence when viewed under uv light).

-Proto. was filtered through a 0.45 μ M filter and pH adjusted with 2 M MOPS to 8.1 for human and *M. xanthus* PPOs, or 8.7 for *B. subtilis* PPO.

Experiments involving fluorescence measurements before and after reduction showed that the fluorescence was always more than 96% complete. The concentration of protoporphyrinogen was thus taken as being equal to that of the protoporphyrin starting solution.

PPO assay procedure

-Substrate was prepared immediately before use.

-100 μ l of the proto. substrate was added to each assay tube, mixed and incubated for 10 min.

-At each time point 100 μ l was transferred from assay tubes into matched "cuvette" tubes (final volume = 1.1 ml) and fluorescence measured.

-Four equivalent time points ranging from 10 min to a maximum of 60 min were used.

-Determination of relative fluorescence units

A customised spreadsheet (Lotus 123) was used. This subtracts the blanks from specimen rates, multiplies by all dilution factors, corrects time to 1 h, and multiply by a regression factor to finally yield enzyme activity in RFUs (nmol/ml/h protoporphyrinogen converted).

Appendix Nine

Inhibition of Protoporphyrinogen Oxidase

(Corrigall et al, 1994)

Reagents and equipment

PPO Inhibitors

- Acifluorfen (AF) and Acifluorfen-methyl (AFM)- (Chem Services West Chester, PA, USA)
- Bilirubin-IX (BR) and Biliverdin-IX hydrochloride- (BV) (Porphyrin Products, Logan USA)
- Haemin (HMN)- (Sigma-Aldridge, St. Louis, MO, USA).

Solvent:

All inhibitors were dissolved in dimethylsulfoxide (DMSO) to give a final solvent concentration of 2.5% in the assay reaction.

Enzyme preparation

- *B. subtilis*, *M. xanthus* and Human PPOs were purified by non-denaturing conditions as outlined in Appendix 1.

Inhibition experiments

- The inhibition assay was performed as previously described in Appendix 7 except for the following modification: the assay buffer was 100 mM Tris/HCl, 3 mM DTT, 1 mM EDTA, 0.1% Tween-20, and pH 8.7 for *B. subtilis* PPO and 8.1 for human and *M. xanthus* PPOs.

IC₅₀ values were determined for the different inhibitors (AF, MeAF, BV, BR, and HMN) by measuring PPO activity at a range of inhibitor concentrations ranging from 0 to 100 μ M at a substrate concentration of 10 μ M. The kinetic constants K_i, K_s, and α were determined by using four different inhibitor concentrations within the range 0 to 10 μ M for each of eight different substrate concentrations ranging from 0.5 to 15 μ M.

Appendix Ten

Production of PPO Antibodies and Western Blotting

(Kirsch et al, 1975)

10.1 Immunization of rabbits and collection of serum

Reagents and equipment

- Immunogen- pure human or *B. subtilis* PPOs (Blotted onto Nitrocellulose membrane) was used for raising the respective antibodies.
- Complete and Incomplete Freund's adjuvants.
- Buffer:-150 mM NaCl, 50 mM Na-phosphate, pH 7.4 (PBS).
- Dimethylsulfoxide (DMSO).
- New Zealand white rabbits

Procedure

Purified *B. subtilis* and human PPO was subjected to SDS-PAGE (see Appendix 2) followed by blotting onto nitrocellulose paper which was then dried and stored at room temperature. When required, membrane containing approximately 100 µg of PPO was cut into small pieces.

-1 ml DMSO was added and whirley mixed to dissolve the nitrocellulose, after which 1.5 ml complete Freund's adjuvant was added and further mixed.

-The immunogen was injected into multiple subcutaneous sites on the back of a rabbit.

-After 3 weeks a booster injection was given with Incomplete Freund's adjuvant (100 µg PPO).

-Booster injections were repeated at 10 day intervals.

-After four inoculations 1 ml of blood was collected from ear vein of rabbit for testing for antibody production by Western blotting. Once an adequate titre was obtained, 30 ml of blood was collected from an ear vein of rabbit.

-The blood was kept at room temperature for 1 h to allow clotting, then stored at 4°C for 2 h before centrifugation at 2 500 G for 30 min. The antibody-containing serum was collected.

-Serum or IgG (see below) was stored at -80°C in small aliquots.

-Specificities of the PPO antisera were checked by Western blotting.

10.2 Isolation of the IgG fraction from antiserum

(Hebert et al, 1973)

Reagents and equipment

Spiramix 10 (Denley, Laboratory and Scientific, Cape Town, S. A.)

Centrifuge (Sorvall RC-3B, The Scientific Co of SA, Johannesburg, South Africa)

PBS pH 7.4

0.9% NaCl pH 8.0 (adjusted with NaOH)

Serum from PPO immunised rabbits

Saturated ammonium sulphate solution

Procedure

-To a given volume of serum, an equal volume of 70% saturated ammonium sulphate was added dropwise whilst gently stirring.

-After standing for 4 h (or overnight) centrifugation was at 3000 G for 15 min.

-The precipitate was resuspended in 0.9% NaCl, pH 8.0; equal to the original serum volume and dissolved by rolling.

-The 70% ammonium sulphate precipitation was repeated, allowing only 30 min standing time prior to centrifugation.

- The resuspension was dialysed against PBS, pH 7.4 overnight (3 X 5 L).

-Absorbance of the solution was read at 280 nm (diluted 5 X) and concentration calculated using the formula:

$$\text{O.D.}_{280} / 1.380 \times \text{dilution factor} = [\text{IgG}] \text{ (mg/ml)}$$

10.3 Western blotting

(Towbin et al, 1979)

Reagents and equipment

-Electroblotting apparatus:-GT Series Gel Electrophoresis Unit with Transphor Power-Lid, model TE 50 (Hoefer Scientific Instruments/Pharmacia Biotech, Cambridge, UK).

-Scotch-Brite pad.

-Power supply.

-Whatman 3M paper.

-0.45 μ M Nitrocellulose membrane (Schleicher & Schuell, Dassel, W. Germany).

-Glass containers for gel and membrane agitation.

-Shaker: The Belly Dancer (Stoval Life Sciences, Inc., Greensboro NC, USA).

Transfer buffer

-4 L of 192 mM glycine, 25 mM Tris plus 1 L of methanol. Chilled to 4°C.

Tris-Buffered Saline (TBS)

-50 mM TrisHCl, 20 mM NaCl, pH 7.4.

Blocking solution

-1% Casein, 0.3% Tween-20 in TBS.

Washing buffer

-0.05% Tween-20 in TBS.

Substrate solution

(made immediately before use).

-24 mg of chloronaphthol was dissolved in 8 ml of methanol, diluted with 40 ml TBS, followed by addition of 100 μ l hydrogen peroxide.

Procedure

Protein transfer onto Nitrocellulose paper

- 3 identical sets of samples of the load, pure PPO and molecular weight markers were run on an SDS polyacrylamide gel.
- Tank was surrounded with ice in a polystyrene container and filled with 5 L of transfer buffer.
- Six pieces of Whatman 3M paper (slightly bigger than the gel size) and a piece of Nitrocellulose membrane (of similar size to the gel) were cut.
- A scotch brite fibre pad was soaked in “transfer buffer” and placed on half of a blotting transfer cassette, followed by three 3 layers of soaked Whatman 3M paper, soaked nitrocellulose membrane, the polyacrylamide gel and finally 3 more layers of Whatman paper. Bubbles between layers were removed with a roller, and the other half of the cassette clipped into place.
- The complete cassette was placed in the tank ensuring that the polyacrylamide gel was closest to the anode. Tank lid (containing the electrodes) was placed on top of the tank in such a way that the cathode faced the operator. Protein transfer was allowed towards the anode at 90 V for 1 h.

Immunodetection

- Current was switched off and assembly removed from tank.
- Nitrocellulose membrane was incubated in blocking solution (200 ml) for 1h.
- The membrane was cut into the 3 identical sections for different primary antibody reactions.
- Membrane sections were incubated with appropriate primary antibody 1/1000 dilution (either IgG or serum) in blocking solution for 1 h. Non-immune rabbit serum or IgG was used in the negative control as the antibodies were raised in rabbits.
- The membranes were incubated with “washing buffer” (3 X 10min).
- Followed by secondary antibody incubation (1/500 dil. goat anti-rabbit peroxidase conjugate) for 1 h.
- Incubation with “wash buffer” (3 X 10 min) was followed by TBS for 10 min.
- The membrane was exposed to substrate solution, with gentle mixing, until proteins were visualised then rinsed in deionised water and dried in the dark.

Appendix Eleven

Immunocharacterisation of Protoporphyrinogen Oxidase

11.1 Immunohistological Localisation of PPO in Different Human Organs

Organ specimens and antibodies

-For light microscopy, surgically removed specimens of human liver, kidney, lung, testis, placenta, ovary, stomach, small and large intestine were fixed in buffered formalin, embedded in paraffin wax and sections cut at 5 microns. Five specimens of each organ were studied.

- Antibody to human PPO was raised as described in Appendix 9. Antibody to Glutathione S-transferase pi was used as a positive control and normal rabbit serum as the negative control. A positive control was included in every experiment and a negative control included for every specimen examined in order to control for nonspecific staining.

Detailed procedure for immunoperoxidase staining

The paraffin sections were brought to absolute alcohol by immersing in

- xylol I (2 min)
- xylol II (2 min)
- absolute alcohol (2 min)

Sections were then:

Incubated with 0.3% H₂O₂ in methanol for 30 min to block endogenous peroxidase, followed by a wash in PBS for 30 min with gentle stirring.

Exposed to normal swine serum (1/20 dilution in PBS) in a humidity chamber for 15 min, after which the swine serum was carefully blotted off.

Exposed to 1/100 dilution in PBS of rabbit anti-PPO (primary antibody) for 30 min at room temperature. A similar dilution of normal rabbit serum was used for the non-immune control, and a 1/500 dilution of Glutathione S-transferase pi antibody was used as a positive control. Sections were washed with PBS for 15 min.

Exposed to 1/250 dilution in PBS of biotinylated swine anti-rabbit immunoglobulins (secondary antibody) for 30 min at room temperature, followed by a wash with PBS for 15 min.

Exposed to 1/500 dilution in PBS of horseradish peroxidase streptavidin for 30 min at RT, followed by a 15 min wash with PBS.

Colour reaction was developed with a solution of 5 mg of 3-3' diaminobenzidine in 10 ml PBS containing 100 μ l of 1% H₂O₂ for approximately 5 min. This was followed by a wash in running tap water for approximately 5 min.

Counter staining of sections was with Meyer's haematoxylin for 10 min followed by a brief rinsing with running tap water, after which section were blued with Scott's tap water for 2 min.

Sections were dehydrated as follows:

- 70% alcohol (2 min)
- 95% alcohol (2 min)
- xylol III (2 min)
- xylol IV (2 min)

and then mounted with Entellen mounting medium. The sections were then viewed under the light microscope.

11.2 Electron Microscopy: Cryoultramicrotomy and Immunogold Labelling

(Monaghan and Atherton, 1992)

Reagents and equipment

1. Reichart Ultracut-S microtome with a Cryochamber
2. KF-80 Rapid Freezing apparatus
3. Aluminium stubs
4. Carbon-coated Formvar-covered Nickel grids
5. Zeiss EM 109 Electron Microscope
6. Specific Antibodies

7. Fixative solution (pH 7.4):
 - 4% paraformaldehyde
 - 0.1% glutaraldehyde
 - 100 mM phosphate buffer
8. 2.3 M Sucrose solution
9. Phosphate buffered saline (PBS) X10, pH 7.4:
10. Bovine Serum Albumin (BSA)
11. Goat anti-rabbit gold complex (5 nm particles)
12. 2% Gelatin/PBS
13. 20 mM Glycine/PBS sterile solution
14. Non-immune normal rabbit serum
15. 25% Glutaraldehyde stock solution
16. Neutral Uranyl Acetate
17. 2% Aqueous Uranyl Acetate
18. 2% Methyl cellulose

Procedure

- Small (0.5-1.0 mm³) tissue specimens were fixed in fixative for approximately 6 h.
- The tissue was gradually infused with sucrose overnight until a final concentration of 2.3 M is reached.
- Tissues were placed in sucrose on aluminium stubs and frozen rapidly in liquid propane at -180⁰C using the KF-80 rapid freezing apparatus.
- Ultra-thin sections (120 nm) were cut from the frozen tissue with a Reichart Ultracut-S microtome, which has a cryochamber maintained at -120⁰C, and the sections collected on sucrose droplets.
- The sections were transferred onto carbon-coated formvar-covered nickel grids and placed face down onto droplets of PBS (2 X 1 min).

-The grids were incubated with 2% gelatin/PBS (3 X 2 min) to reduce non-specific labelling followed by incubation with 20 mM glycine/PBS (1 X 10 min) to quench residual aldehydes.

-The grids were washed 1% BSA/PBS (5 X 1 min).

-The sections were then incubated overnight at room temperature with primary antibody (1/50 dilution of PPO polyclonal antibody raised in rabbits). Control sections were incubated with same dilution of non-immune normal rabbit serum.

-Grids were rinsed with 1% BSA/PBS (10 X 2 min).

-Sections were incubated with goat anti-rabbit gold complex, 5 nm particles, 1/30-1/60 dilution in 1% BSA/PBS at room temperature for 1 h.

-Grids were rinsed with 1% BSA/PBS (5 X 1 min), followed by PBS (5 X 1 min).

-The gold particles were immobilised with 1% glutaraldehyde/PBS (1 X 5 min).

-Grids were rinsed with distilled water (5 X 2 min).

-The sections were incubated with neutral uranyl acetate (1 X 10 min).

-Grids were rinsed with distilled water (2 X 1 min).

-The grids were coated with a mixture of 1:9 of 2% uranyl acetate and 2% methyl cellulose at 4°C (UA/MC) (2 X 2.5 min), picked up with wire loops and the excess UA/MC removed by scraping over blotting paper.

-Air-drying at room temperature followed.

-The tissue sections on the grids were examined by electron microscopy at 80 kV.

REFERENCES

References

- Abdulla, M. and Haeger-Aronsen, B. (1971). ALA dehydratase activation by zinc. *Enzyme* 12: 708-710.
- Adler, A. D. Ed. (1973). The chemical and physical behaviour of porphyrin compounds and related structures. *Ann. N. Y. Acad. Sci.* 206: 1-761.
- Al-Karadaghi, S., Hansson, M., Nikonov, S., Jonsson, B. and Hederstedt, L. (1997). Crystal structure of ferrochelatase the terminal enzyme in heme biosynthesis. *Structure* 5 (11): 1501-1510.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215: 403-410.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs". *Nucl. Acid Res.* 25: 3389-3402.
- Alwan, A. F. and Jordan, P. M. (1988). Isolation of uroporphyrinogen III synthase from *Escherichia coli*. *Biochem. Soc. Trans.* 16: 965-966.
- Amillet, J. M. and Labbe-Bois, R. (1995). Isolation of the gene *HEM4* encoding uroporphyrinogen III synthase in *Saccharomyces cerevisiae*. *Yeast* 11 (5): 419-424.
- Anderson, P. M. and Desnick, R. J. (1979). Purification and properties of δ -aminolevulinic acid dehydratase from human erythrocytes. *J. Biol. Chem.* 254: 6924-6930.
- Anderson, P. M. and Desnick, R. J. (1980). Purification and properties of uroporphyrinogen I synthase from human erythrocytes. *J. Biol. Chem.* 255: 1993-1999.
- Anderson, P. M., Reddy, R. M., Anderson, K. E. and Desnick, R. J. (1981). Characterization of the porphobilinogen deaminase deficiency in acute intermittent porphyria. *J. Clin. Invest.* 68: 1-12.
- Anderson, R. J., Norris, A. E. and Hess, F. D. (1994). Synthetic organic chemicals that act through the porphyrin pathway. *Amer. Chem. Soc. Symp. Ser.* 559: 18-33.
- Arnould, S., Berthon, G-L., Hubert, C., Dias, M., Cibert, C., Mornet, R. and Camadro, J-M. (1997). Kinetics of protoporphyrinogen oxidase inhibition by diphenyleneiodonium derivatives. *Biochem.* 36: 10178-10184.
- Arnould, S., Takahashi, M. and Camadro, J-M. (1998). Stability of recombinant yeast protoporphyrinogen oxidase: effect of diphenyl ether-type herbicides and diphenyleneiodonium. *Biochem.* 37 (37): 12818-12828.
- Arnould, S., Takahashi, M., and Camadro, J. M. (1999). Acylation stabilizes a protease-resistant conformation of protoporphyrinogen oxidase, the molecular target of diphenyl ether-type herbicides. *Proc. Natl. Acad. Sci. USA* 96 (26): 14825-14830.

- Astrin, K. H., Warner, C. H., Yoo, H. W., Goodfellow, P. J., Tsai, S. F. and Desnick, R. J. (1991). Regional assignment of the human uroporphyrinogen III synthase (UROS) gene to chromosome 10q25.2---q26.3. *Hum. Genet.* 87 (1): 18-22.
- Awan, S. J., Siligardi, G., Shoolingin-Jordan, P. M. and Warren, M. J. (1997). Reconstitution of the holoenzyme form of *Escherichia coli* porphobilinogen deaminase from apoenzyme with porphobilinogen and preuroporphyrinogen: a study using circular dichroism spectroscopy. *Biochem.* 36 (30): 9273-9282.
- Ayala, F. J. and Kiger, J. A. Jr (1980). Speciation and macroevolution, Chap. 22. In: *Modern Genetics*, 1st ed., The Benjamins/Cummins Publishing Co., Inc., California, USA, pp. 730-780.
- Barnes, H. D. (1945). A note on porphyria with a resume of eleven South African cases. *Clin. Proc.* 4: 269-275.
- Barnes, H. D. (1951). Further South African cases of porphyria. *S. Afr. J. Clin. Sci.* 2: 117-169.
- Battle, A. M. del C., Benson, A. and Rimington, C. (1965). Purification and properties of coproporphyrinogenase. *Biochem. J.* 97: 731-740.
- Battersby, A. R. and Macdonald, E. (1975). Biosynthesis of porphyrins, chlorins and corrins. In: *Porphyrins and metalloporphyrins*, Smith, K. M. ed., Elsevier Amsterdam, 61-122.
- Battersby, A. R., Fookes, C. J. R., Hart, G., Matcham, G. W. J. and Macdonald, E. (1979). Order of assembly of the four pyrrole rings during biosynthesis of natural porphyrins. *J. Chem. Soc. Res. Commun.* 539-541.
- Battersby, A. R., Fookes, C. J. R., Matcham, G. W. J., Macdonald, E. and Hollenstein, R. (1983). Biosynthesis of porphyrins and related macrocycles. Part 20. Purification of deaminase and studies on its mode of action. *J. Chem. Soc. Perkin Trans. 1*: 3031-3040.
- Baumstark, F. (1874). Zwei pathologische harnfarbstoffe. *Pflügers Arch. Gest. Physiol.* 9: 568-584.
- Bawden, M. J., Borthwick, I. A., Healy, H. M., Morris, C. P., May, B. K. and Elliott, W. H. (1987). Sequence of 5-aminolevulinic acid synthase cDNA. *Nucl. Acid Res.* 15: 8563.
- Beale, S. I. and Castelfranco, P. A. (1974a). *Plant Physiol.* 53: 291-296.
- Beale, S. I. and Castelfranco, P. A. (1974b). *Plant Physiol.* 53:297-303.
- Beale, S. I. and Weinstein, J. D. (1990). Tetrapyrrole metabolism in photosynthetic organisms. In: *Biosynthesis of heme and chlorophyll*, Dailey, H. A. eds. McGraw-Hill, New York, 287-391.
- Beale, S. I., Gough, S. P. and Granick, S. (1975). Biosynthesis of delta-aminolevulinic acid from the intact carbon skeleton of glutamic acid in greening barley. *Proc. Natl. Acad. Sci. USA* 72: 2719.
- Beaumont, C., Foubert, C., Grandchamp, B., Weil, D., Van Cong N'Guyen, Gross, M. S. and Nordmann, Y. (1984). Assignment of the human gene for ALA dehydratase to chromosome 9 by somatic cell hybridisation and specific enzyme immunoassay. *Ann. Hum. Genet.* 48: 153-159.
- Berzelius, J. J. (1840). *Lerbuch der Chemie*, Arnoldische Buchhandlung; Dresden Leipzig 9: 67-69.

- Bevan, D. R., Bodlaender, P. and Shemin, D. (1980). Mechanism of porphobilinogen synthase. Requirement of Zn^{2+} for enzyme activity. *J. Biol. Chem.* 255 (5): 2030-2035.
- Birchfield, N. B. and Casida, J. E. (1996). Protoporphyrinogen oxidase: High affinity tetrahydrophthalimide radioligand for the inhibitor/herbicide-binding site in mouse liver mitochondria. *Chem. Res. Toxicol.* 9: 1135-1139.
- Birchfield, N. B., Latli, B. and Casida, M. (1998). Human protoporphyrinogen oxidase: Relation between the herbicide binding site and the flavin cofactor. *Biochem.* 37: 6905-6910.
- Bishop, D. F. (1990). Two different genes encode delta-aminolevulinate synthase in humans: nucleotide sequence of cDNAs for the housekeeping and erythroid genes. *Nucleic Acid Res.* 18: 7187-7188.
- Bishop, D. F., Henderson, A. H. and Astrin, K. H. (1990). Human δ -aminolevulinate synthase: Assignment of the housekeeping gene to chromosome 3p21 and the erythroid-specific gene to the X chromosome. *Genomics* 7 (2): 207-214.
- Bishop, T. R., Miller, M. W., Beall, J., Zon, L. I. and Dierks, P. (1996). Genetic regulation of δ -aminolevulinic dehydratase during erythropoiesis. *Nucleic Acid Res.* 24 (13): 2511-2518.
- Bissbort, S., Hitzeroth, H. W., du Wentzel, D. P., Van den Berg, C. W., Senff, H., Wienker, T. F. and Bender, K. (1988). Linkage between the variegate porphyria (VP) and the alpha-1 antitrypsin (PI) genes on human chromosome 14. *Hum. Genet.* 79: 289-290.
- Bissell, D. M. and Schmid, R. (1987). Hepatic porphyrias. In: *Diseases of the liver*, Schiff, L. and Schiff, E. R. 6th ed., J. B. Lippincott, Philadelphia, Toronto, pp 1075-1092.
- Blake, D., McManus, J., Cronin, V. and Ratnaik, S. (1992). Faecal coproporphyrin isomers in hereditary coproporphyria. *Clin Chem.* 38: 96-100.
- Bloomer, J. R. and Pierach, C. A. (1982). Effect of hematin administration to patients with protoporphyria and liver disease. *Hepatology* 2: 817-821.
- Boese, Q. F., Spano, A. J., Li, J. M. and Timko, M. P. (1991). Aminolevulinic acid dehydratase in pea (*Pisum sativum* L.). Identification of an unusual metal-binding domain in the plant enzyme. *J. Biol. Chem.* 266 (26): 17060-17066.
- Bogard, M., Camadro, J.-M., Nordmann, Y. and Labbe, P. (1989). Purification and properties of mouse liver coproporphyrinogen oxidase. *Eur. J. Biochem.* 181 (2): 412-421.
- Bogorad, L. (1955). *Science* 121: 878-879.
- Borthwick, I. A., Srivastava, G., Booker, J. D., May, B. K. and Elliot, W. H. (1983). Purification of 5-aminolaevulinate synthase from liver mitochondria of chick embryo. *Eur. J. Biochem.* 129 (3): 615-620.
- Borthwick, I. A., Srivastava, G., Day, A. R., Pirola, B. A., Snowswell, M. A., May, B. K. and Elliott, W. H. (1985). Complete nucleotide sequence of 5-aminolaevulinate synthase precursor. *Eur. J. Biochem* 150 (3): 481-484.

- Bottomley, S. S. and Muller-Eberhard, U. (1988). Pathophysiology of heme synthesis. *Semin. in Hematol.* 25: 282-302.
- Boyle, J., Hordovatz, X., Thompson, G. G., and Moore, M. R. (1986). The use of leucocyte protoporphyrinogen oxidase activity in screening a family with variegate porphyria. *Biochem. Soc. Trans.* 14: 153-154.
- Bradford, M. M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
- Bradshaw, R. E., Dixon, S. W., Raitt, D. C. and Pillar, T. M. (1993). Isolation and nucleotide sequence of the 5-aminolevulinic acid synthase gene from *Aspergillus nidulans*. *Current Genet.* 23 (5-6): 501-507.
- Brenner, D. A. and Bloomer, J. R. (1980a). The enzymatic defect in variegate porphyria. *N. Engl. J. Med.* 302: 765-769.
- Brenner, D. A. and Bloomer, J. R. (1980b). A fluorometric assay for measurement of protoporphyrinogen oxidase activity in mammalian tissue. *Clin. Chim. Acta* 100: 259-266.
- Brenner, D. A. and Frasier, F. (1991). Cloning of murine ferrochelatase. *Proc Natl. Acad. Sci. USA* 88 (3): 849-853.
- Brenner, D. A., Didier, J. M., Frasier, F., Christensen, S. R., Evans, G. A. and Dailey, H. A. (1992). A molecular defect in human protoporphyrin. *Am. J. Hum. Genet.* 50 (6): 1203-1210.
- Breu, V. and Dornemann, D. (1988). Formation of 5-aminolaevulinic acid via glutamate-1-semialdehyde and 4,5-dioxovalerate with participation of an RNA component in *Scenedesmus obliquus* mutant C-2A'. *Biochem. Biophys. Acta* 967: 135-140.
- Breu, V., Kah, H. and Dornemann, D. (1988). *Biochem. Biophys. Acta* 964: 61-68.
- Brodie, M. J., Moore, M. R., Thompson, G. G. and Goldberg, A. (1977a). The treatment of acute intermittent porphyria with laevulose. *Clin. Sci. Mol. Med.* 53: 365-371.
- Brodie, M. J., Thompson, G. G., Moore, M. R., Beattie, A. D. and Goldberg, A. (1977b). Hereditary coproporphyrin. *Quart. J. Med.* 46: 229-241.
- Bruyant, P. and Kannangara, C. G. (1987). Biosynthesis of 5-ALA in greening barley leaves. VIII. Purification and characterization of glutamate tRNA ligase. *Carlsberg Res. Comm.* 52: 99-109.
- Burnham, B. (1968). The chemistry of the porphyrins. *Semin. Hematol.* 5: 296-322.
- Cacheux, V., Martasek, P., Fougereuse, F., Delfau, M. H., Druart, L., Tachdian, G. and Grandchamp, B. (1994). Localization of the human coproporphyrinogen oxidase gene to chromosome band 3q12. *Hum. Genet.* 94 (5): 557-559.
- Camadro, J.-M., Thome, F., Brouillet, N., and Labbe, P. (1994). Purification and properties of protoporphyrinogen oxidase from the yeast *Saccharomyces cerevisiae*. Mitochondrial location and evidence for a precursor form of the protein. *J. Biol. Chem.* 269 (51): 32085-32091.

- Camadro, J.-M. and Labbe, P. (1996). Cloning and characterization of the yeast *HEM14* gene coding for protoporphyrinogen oxidase, the molecular target of diphenyl ether-type herbicides. *J. Biol. Chem.* 271 (15): 9120-9128.
- Camadro, J.-M., Abraham, N. G. and Levere, R. D. (1985). Kinetic properties of the membrane-bound human liver mitochondrial protoporphyrinogen oxidase. *Arch. Biochem. Biophys.* 242 (1): 206-212.
- Camadro, J.-M., Chambon, H., Jolles, J. and Labbe, P. (1986). Purification and properties of coproporphyrinogen oxidase from the yeast *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 156 (3): 579-587.
- Camadro, J.-M., Matringe, M., Scalla, R. and Labbe, P. (1991). Kinetic studies on protoporphyrinogen oxidase inhibition by diphenyl ether herbicides. *Biochem. J.* 277: 17-21.
- Campbell, J. A. H., Corrigall, A. V., Guy, A. and Kirsch, R. E. (1991). Immunohistologic localization of Alpha, Mu and Pi class glutathione S-transferases in human tissues. *Cancer* 67: 1608-1613.
- Cardalda, C. A., Batlle, A. and Juknat, A. A. (1998). Sequence and structure of the rat housekeeping PBG-D isoform. *Biochem. Biophys. Res. Commun.* 249 (2): 438-443.
- Cerleti, P. and Giordano, M. G. (1971). Determination of flavin compounds in tissues. *Methods Enzymol.* 18B: 285-290.
- Chauhan, S. and O'Brian, M. R. (1993). *Bradyrhizobium japonicum* delta-aminolevulinic acid dehydratase is essential for symbiosis with soybean and contains a metal-binding domain. *J. Bacteriol.* 175 (22): 7222-7227.
- Choi, K. W., Han, O. H., Lee, H. J., Yun, Y. H., Moon, Y. H., Kim, M., Kuk, Y. I., Han, S. U., and Guh, J. O. (1998). Generation of resistance to the diphenyl-ether herbicide, oxyfluorfen, via expression of the *Bacillus subtilis* protoporphyrinogen oxidase gene in transgenic tobacco plants. *Biosci. Biotechnol. Biochem.* 62: 558-560.
- Chretien, S., Dubart, A., Beaupain, D., Raich, A., Grandchamp, B., Goossens, M. and Romeo, P. H. (1988). Alternative transcription and splicing of the human porphobilinogen deaminase gene results in tissue-specific or in housekeeping expression. *Proc. Natl. Acad. Sci. USA* 85: 6-10.
- Coakley, J., Blake, D., Hawkins, R., Nordmann, Y., Crinis, N., Sloan, L., McManus, J. and Connelly, J. (1990). An unusual case of variegate porphyria with possible homozygous inheritance. *Australian and New Zealand J. Med.* 20: 587-589.
- Conboy, J. G., Cox, T. C., Bottomley, S. S., Bawden, M. J. and May, B. K. (1992). Human erythroid 5-aminolevulinate synthase. Gene structure and species-specific differences in alternative RNA splicing. *J. Biol. Chem.* 267 (26): 18753-18758.
- Conder, L. H., Woodard, S. I., and Dailey, H. A. (1991). Multiple mechanisms for the regulation of haem synthesis during erythroid cell differentiation. Possible role for coproporphyrinogen oxidase. *Biochem. J.* 275: 321-326.
- Cookson, G. H. and Rimington, C. (1954). Porphobilinogen. *Biochem. J.* 57: 476-484.

- Coomber, S. A., Jones, R. M., Jordan, P. M. and Hunter, C. N. (1992). A putative anaerobic coproporphyrinogen III oxidase in *Rhodobacter sphaeroides*. I. Molecular cloning, transposon mutagenesis and sequence analysis of the gene. *Molec. Microbiol.* 6: 3155-3169.
- Corrigall, A. V., Hift, R. J., Adams, P. A. and Kirsch, R. E. (1994). Inhibition of mammalian protoporphyrinogen oxidase by acifluorfen. *Biochem. Mol. Biol. Internat.* 34 (6): 1283-1289.
- Corrigall, A. V., Hift, R. J., Davids, L. M., Hancock, V., Meissner, D., Kirsch, R. E., and Meissner, P. N. (2000). Homozygous variegate porphyria in South Africa: genotypic analysis in two cases. *Mol. Genet. Metab.* 69 (4): 323-330.
- Corrigall, A. V., Hift, R. J., Hancock, V., Meissner, D., Davids, L., Kirsch, R. E. and Meissner, P. N. (1998). Identification and characterisation of a deletion (537delAT) in the protoporphyrinogen oxidase gene in a South African variegate porphyria family. *Hum. Mutat.* 12 (6): 403-407.
- Corrigall, A. V., Meissner, P. N. and Kirsch, R. E. (1991). Purification of human erythrocyte porphobilinogen deaminase. *S. Afr. Med. J.* 80 (6): 294-296.
- Cotter, P. D., Baumann, M. and Bishop, D. F. (1992). Enzymatic defect in "X-linked" sideroblastic anemia: molecular evidence for erythroid delta-aminolevulinate synthase deficiency. *Proc. Natl. Acad. Sci. USA* 89 (9): 4028-4032.
- Cotter, P. D., May, A., Fitzsimons, E. J., Houston, T., Woodcock, B. E., Al-Sabah, A. I., Wong, L. and Bishop, D. F. (1995). Late-onset X-linked sideroblastic anemia. Missense mutations in the erythroid delta-aminolevulinate synthase (ALAS2) gene in two pyridoxine-responsive patients initially diagnosed with acquired refractory anemia and ringed sideroblasts. *J. Clin. Invest.* 96 (4): 2090-2096.
- Cotter, P. D., May, A., Li, L., Al-Sabah, A. I., Fitzsimons, E. J., Cazzola, M. and Bishop, D. F. (1999). Four new mutations in the erythroid-specific 5-aminolevulinate synthase (ALAS2) gene causing X-linked sideroblastic anemia: Increased pyridoxine responsiveness after removal of iron overload by phlebotomy and co-inheritance of hereditary hemochromatosis. *Blood* 93 (5): 1757-1759.
- Cotter, P. D., Rucknagel, D. L. and Bishop, D. F. (1994). X-linked sideroblastic anemia: identification of the mutation in the erythroid-specific delta-aminolevulinate synthase gene (ALAS2) in the original family described by Cooley. *Blood* 84 (11): 3915-3924.
- Cox, T. C., Bawden, M. J., Martin, M. J. and May, B. K. (1991). Human erythroid 5-aminolevulinate synthase: promoter analysis and identification of an iron responsive element. *EMBO J.* 10 (7): 1891-1902.
- Cox, T. M., Graeme, M. D., Alexander, J. M. and Sarkany, R. P. E. (1998). Protoporphyria. *Semin. Liv. Dis.* 18 (1): 85-93.
- Creighton, C. E. (1993). Evolutionary and genetic origin of protein sequences, Chap. 3. In: *Proteins - Structures and molecular properties*, 2nd ed., W. H. Freeman and Co., New York, pp. 105-138.
- Crouse, B. R., Sellers, V. M., Finnegan, M. G., Dailey, H. A. and Johnson, M. K. (1996). Site-directed mutagenesis and spectroscopic characterization of human ferrochelatase: identification of residues coordinating the [2Fe-2S] cluster. *Biochem.* 35 (50): 16222-16229.

- Da Silva, V., Simoni, S., Deybach, J-C., Puy, H. and Nordmann, Y. (1995). Variegate porphyria: diagnostic value of fluorometric scanning of plasma porphyrins. *Clin Chim. Acta* 238: 163-168.
- Dailey, H. A. (1982). Purification and characterization of membrane-bound ferrochelatase from *Rhodopseudomonas sphaeroides*. *J. Biol. Chem.* 257 (24): 14714-14718.
- Dailey, H. A. (1990). Conversion of coproporphyrinogen to protoheme in higher eukaryotes and bacteria: terminal three enzymes. In: Biosynthesis of heme and chlorophylls, Dailey, H. A. ed., New York, McGraw-Hill, pp. 123-162.
- Dailey, H. A. and Dailey, T. A. (1996a). Protoporphyrinogen oxidase of *Myxococcus xanthus*. Expression, purification, and characterization of the cloned enzyme. *J. Biol. Chem.* 271 (15): 8714-8718.
- Dailey, H. A. and Dailey, T. A. (1997b). Characterization of human protoporphyrinogen oxidase in controls and variegate porphyria. *Cell Molec. Biol.* 43: 67-73.
- Dailey, H. A. and Fleming, J. E. (1983). Bovine ferrochelatase. Kinetic analysis of inhibition by N-methylprotoporphyrin, manganese, and heme. *J. Biol. Chem.* 258 (19): 11453-11459.
- Dailey, H. A., and Karr, S. W. (1987). Purification and characterization of murine protoporphyrinogen oxidase. *Biochem.* 26: 2697-2701.
- Dailey, H. A., Finnegan, M. G. and Johnson, M. K. (1994c). Human ferrochelatase is an iron-sulfur protein. *Biochem.* 33 (2): 403-407.
- Dailey, H. A., Sellers, V. M. and Dailey, T. A. (1994b). Mammalian ferrochelatase. Expression and characterization of normal and two human porphyric ferrochelatases. *J. Biol. Chem.* 269 (1): 390-395.
- Dailey, T. A. and Dailey, H. A. (1997a). Expression, purification and characterization of mammalian protoporphyrinogen oxidase. *Methods Enzymol.* 281: 340-349.
- Dailey, T. A. and Dailey, H. A. (1998). Identification of an FAD superfamily containing protoporphyrinogen oxidases, monoamine oxidases, and phytoene desaturases. Expression and characterization of phytoene desaturase of *Myxococcus xanthus*. *J. Biol. Chem.* 273 (22): 13658-13662.
- Dailey, T. A., and Dailey, H. A. (1996b). Human protoporphyrinogen oxidase: Expression, purification and characterization of the cloned enzyme. *Prot. Sci.* 5: 98-105.
- Dailey, T. A., Dailey, H. A., Meissner, P. N., and Prasad, A. R. K. (1995). Cloning, sequencing, and expression of mouse protoporphyrinogen oxidase. *Arch. Biochem. Biophys.* 324 (2): 379-384.
- Dailey, T. A., Meissner, P., and Dailey, H. A. (1994a). Expression of a cloned protoporphyrinogen oxidase. *J. Biol. Chem.* 269: 813-815.
- Davies, R. C. and Neuberger, A. (1973). Polypyrroles formed from porphobilinogen and amines by uroporphyrinogen synthetase of *Rhodopseudomonas sphaeroides*. *Biochem. J.* 133: 471-492.
- Day, A. L., Parsons, B. M. and Dailey, H. A. (1998). Cloning and characterization of *Gallus* and *Xenopus* ferrochelatases: presence of the [2Fe-2S] cluster in the nonmammalian ferrochelatase. *Arch. Biochem. Biophys.* 359 (2): 160-169.

- Day, R. S. (1986). Variegate porphyria. *Semin. Dermatol.* 5: 138-154.
- Day, R. S. and Eales, L. (1982). Porphyrins in renal transplantation. *Nephron* 30: 22-27.
- Day, R. S., Eales, L., and Meissner, D. (1982). Co-existent variegate porphyria and porphyria cutanea tarda. *N. Engl. J. Med.* 307: 36-41.
- Day, R. S., Pimstone, N. R. and Eales, L. (1978). The diagnostic value of blood plasma porphyrin methyl ester profiles produced by quantitative TLC. *Int. J. Biochem.* 9: 897-904.
- Dayan, F. E. and Duke, S. O. (1997a). Overview of Protoporphyrinogen Oxidase-Inhibiting Herbicides. In: *The 1997 Brighton Crop Protection Conference- Weeds*, pp. 83-92.
- Dayan, F. E. and Duke, S. O. (1997b). Phytotoxicity of protoporphyrinogen oxidase inhibitors: Phenomenology, mode of action and mechanism of resistance. In: *Herbicide activity: Toxicology, biochemistry and molecular biology*, Roe, R. M., Burton, J. D. and Kuhr, R. J. eds. I.O.S. Press Inc., Amsterdam, pp. 11-35.
- Dayan, F. E. and Duke, S. O. (1997c). Porphyrin-generating Herbicides. In: *Herbicides*. United States Department of Agriculture- Agricultural Research Services, USA.
- Dayan, F. E., Duke, S. O., Reddy, K. N., Hamper, B. C. and Leschinsky, K. L. (1997a). Effects of isoxazole herbicides on protoporphyrinogen oxidase and porphyrin physiology. *J. Agric. Food Chem.* 45: 967-975.
- Dayan, F. E., Duke, S. O., Weete, J. D. and Hancock, H. G. (1997b). Selectivity and mode of action of carfentrazone-ethyl, a novel phenyl triazolinone herbicide. *Pestic. Sci.* 51: 65-73.
- Dayan, F. E., Weete, J. D. and Hancock, H. G. (1996). Physiological basis for differential sensitivity to sulfentrazone by sicklepod (*Senna obtusifolia*) and coffe senna (*Cassia occidentalis*). *Weed Sci.* 44: 12-17.
- De Barreiro, O. L. (1967). 5-aminolaevulinate hydrolyase from yeast. Isolation and purification. *Biochim. Biophys. Acta* 139 (2): 479-486.
- de Verneuil, H., Aitken, G. and Nordmann, Y. (1978). Familial and sporadic porphyria cutanea tarda: two different diseases. *Hum. Genet.* 44: 145-151.
- de Verneuil, H., Sassa, S. and Kappas, A. (1983). Purification and properties of uroporphyrinogen decarboxylase from human erythrocytes - a single enzyme catalysing the four sequential decarboxylations of uroporphyrinogens I and III. *J. Biol. Chem.* 258: 2454-2460.
- Dean, G. (1953). Porphyria. *Br. Med. J.* 2: 1291-1294.
- Dean, G. (1957). *Scientific Amer.* 196: 133.
- Dean, G. (1971). *The Porphyrrias: A Story of Inheritance and Environment*, 2d ed., Pitman Medical, London.
- Dean, G. and Barnes, H. D. (1958). Porphyria. A South African screening experiment. *Br. Med. J.* 1: 298-301.
- Dean, G. and Barnes, H. D. (1959). Porphyria in Sweden and South Africa. *S. Afr. Med. J.* 33: 246-253.

- Delfau-Larue, M. H., Martasek, P. and Grandchamp, B. (1994). Coproporphyrinogen oxidase: gene organisation and description of a mutation leading to exon 6 skipping. *Hum. Molec. Genet.* 3 (8): 1325-1330.
- Desnick, R. J., Glass, I. A., Xu, W., Solis, C. and Astrin, K. H. (1998). Molecular genetics of congenital erythropoietic porphyria. *Semin. Liv. Dis.* 18 (1): 77-84.
- Desnick, R. J., Ostaciewicz, L., Tischler, P. and Mustajoki, P. (1985). Acute intermittent porphyria: characterisation of a novel mutation in the structural gene for porphobilinogen deaminase. Demonstration of non-active enzyme intermediates stabilized by bound protein. *J. Clin. Invest.* 76: 865-874.
- Devine, M. D., Duke, S. O. and Fedke, C. (1993). Oxygen toxicity and herbicidal action. In: *Physiology of herbicidal action*, Prentice Hall, Englewood Cliffs, New Jersey, pp. 177-189.
- Devlin, R. S., Karczmarczyk, S. J. and Zbiee, I. I. (1983). Influence of norflurazon on the activation of substituted diphenyl ether herbicides by light. *Weed Sci.* 31: 109-112.
- Deybach, J-C. and Puy, H. (1995). Porphobilinogen deaminase gene structure and molecular defects. *J. Bioenergetics & Biomembranes* 27(2): 197-205.
- Deybach, J-C., da Silva, V., Grandchamp, B. and Nordmann, Y. (1985). The mitochondrial location of protoporphyrinogen oxidase. *Eur. J. Biochem.* 149 (2): 431-435.
- Deybach, J-C., de Verneuil, H. and Nordmann, Y. (1981). The inherited defect in porphyria variegata. *Hum. Genet.* 58: 425-428.
- Deybach, J-C., Puy, H., Robreau, A-M., Lamoril, J., da Silva, V, Grandchamp, B. and Nordmann, Y. (1996). Mutations in the protoporphyrinogen oxidase gene in patients with variegate porphyria. *Hum. Molec. Genet.* 5 (3): 407-410.
- Diflumeri, C., Larocque, R. and Keng, T. (1993). Molecular analysis of *HEM6 (HEM12)* in *Saccharomyces cerevisiae*, the gene for uroporphyrinogen decarboxylase. *Yeast* 9 (6): 613-623.
- Dolphin, D. (1979). *The Porphyrins* 7 vols, Academic Press, New York Eds.
- Doss, M., Schneider, J., von Tiepermann, R. and Brandt, R. (1982). A new type of acute porphyria with porphobilinogen synthase defect in the homozygous state. *Clin. Biochem.* 15: 52-55.
- Doussiere, J. and Vignais, P. V. (1992). Diphenylene iodonium is an inhibitor of the NADPH oxidase complex of bovine neutrophils. Factors controlling the inhibitory potency of diphenylene iodonium in a cell-free system of oxidase activation. *Eur. J. Biochem.* 208 (1): 61-71.
- Drew, P. D. and Ades, I. Z. (1989). Regulation of the stability of chicken embryo liver delta-aminolevulinate synthase mRNA by hemin. *Biochem. Biophys. Res. Commun.* 162: 102-107.
- Dubart, A., Mattei, M. G., Raich, N., Beaupain, D., Romeo, P. -H., Mattei, J. F. and Goossens, M. (1986). Assignment of human uroporphyrinogen decarboxylase (URO-D) to the p34 band of chromosome 1. *Hum. Genet.* 73 (3): 277-279.

- Duke, S. O., Becerril, J. M., Matsumoto, H. and Sherman, T. D. (1991). Photosensitizing porphyrins as herbicides. *Amer. Chem. Soc. Symp. Ser.* 449: 371-386.
- Duke, S. O., Lee, H. J., Duke, M. V., Reddy, K. N., Sherman, T. D., Beceril, J. M., Nandihalli, U. B., Matsumoto, H., Jacobs, N. J. and Jacobs, J. M. (1997). Mechanisms of resistance to protoporphyrinogen oxidase-inhibiting herbicides. In: *Weed and crop resistance to herbicides*, De Prado, R., Jorin, J. and Garcia-Torres, L. eds. Kluwer Academic Publishers, Amsterdam, pp. 155-160.
- Duke, S. O., Lee, H. J., Nandihalli, U. B. and Duke, M. V. (1994). Protoporphyrinogen oxidase as optimal herbicide target in the porphyrin pathway. *Amer. Chem Soc. Symp. Ser.* 559: 191-204.
- Duke, S. O., Vaughn, K. C. and Meusen, R. L. (1984). Mitochondrial involvement in the mode of action of acifluorfen. *Pestic. Biochem. Physiol.* 21: 368-376.
- Dzelzkalns, V., Foley, T. and Beale, I. (1982). Delta-aminolevulinic acid synthase of *Euglena gracilis*: physical and kinetic properties. *Arch. Biochem. Biophys.* 216 (1): 196-203.
- Eales, L. (1961). The porphyrias. *Ann. Rev. Med.* 12: 251-270.
- Eales, L. (1963). Porphyria as seen in Cape Town. A survey of 250 patients and some recent studies. *S. Afr. J. Lab. Clin. Med.* 9: 151-161.
- Eales, L. and Chait, J. (1939). A case of idiopathic porphyria. *Inyanga (I)* 14: 39-53; Publication of the University of Cape Town Medical Faculty.
- Eales, L., Day, R. S. and Blekkenhorst, G. H. (1980). The clinical and biochemical features of variegate porphyria: an analysis of 300 patients studied at Groote Schuur Hospital, Cape Town. *Int. J. Biochem.* 12: 837-853.
- Echelard, Y., Demetryszyn, J., Drolet, M. and Sasarman, A. (1988). Nucleotide sequence of the *hemB* gene of *Escherichia coli* K12. *Mol. Gen. Genet.* 214 (3): 503-508.
- Edgar, A. J. and Wickramasinghe, S. N. (1998). Hereditary sideroblastic anaemia due to a mutation in exon 10 of the erythroid 5-aminolaevulinate synthase gene. *Br. J. Haematol.* 100 (2): 389-392.
- Edgar, A. J., Losowsky, M. S., Noble, J. S. and Wickramasinghe, S. N. (1998). Identification of an arginine 452 to histidine substitution in the erythroid 5-aminolaevulinate synthase gene in a large pedigree with X-linked hereditary sideroblastic anaemia. *Eur. J. Haematol.* 58 (1): 1-4.
- Edgar, A. J., Vidyatilake, H. M. and Wickramasinghe, S. N. (1998). X-linked sideroblastic anaemia due to a mutation in the erythroid 5-aminolaevulinate synthase gene leading to an arginine 170 to leucine substitution. *Eur. J. Haematol.* 61 (1): 55-58.
- Eiberg, H., Mohr, J. and Staub-Nielsen, I. (1983). Delta-aminolevulinate dehydratase - syntenicity with ABO-AKI-ORM and assignment to chromosome 9. *Clin. Genet.* 23: 150-154.
- Elder, G. H. (1982). Enzymatic defects in porphyria: An overview. *Semin. Liv. Dis.* 2: 87-99.
- Elder, G. H. (1993). Molecular genetics of disorders of haem biosynthesis. *J. Clin. Pathol.* 46: 977-981.
- Elder, G. H. (1998). Porphyria cutanea tarda. *Semin. Liv. Dis.* 18 (1): 67-75.

- Elder, G. H. and Evans, J. O. (1978). Evidence that the coproporphyrinogen oxidase activity of rat liver is situated in the intermembrane space of mitochondria. *Biochem. J.* 172 (2): 345-347.
- Elder, G. H. and Roberts A. G. (1994). Uroporphyrinogen decarboxylase. *J. Bioenerg. Biomembr.* 27 (2): 207-214.
- Elder, G. H., Evans, J. O., Thomas, N., Cox, R., Brodie, M. J., Moore, M. R., Goldberg, A and Nicholson, D. C. (1976). The primary enzyme defect in hereditary coproporphyria. *Lancet* 2: 1217-1219.
- Elder, G. H., Hift, R. J. and Meissner, P. N. (1997). The acute porphyrias. *Lancet* 349: 1613-1617.
- Elder, G. H., Roberts, A. G. and De Salamanca, R. E. (1989). Genetics and pathogenesis of human uroporphyrinogen decarboxylase defects. *Clin. Biochem.* 22: 163-168.
- Elder, G. H., Tovey, J. A. and Sheppard, D. M. (1983). Purification of uroporphyrinogen decarboxylase from human erythrocytes. *Biochem. J.* 215: 45-55.
- Elder, G. H., Urquhart, A. J, de Salamanca, R. E., et al (1985). Immunoreactive uroporphyrinogen decarboxylase in the liver in porphyria cutanea tarda. *Lancet* 2: 229-232.
- Eldridge, M. G. and Dailey, H. A. (1992). Yeast ferrochelatase: expression in a baculovirus system and purification of the expression protein. *Prot. Sci.* 1 (2): 221-227.
- Elliott, T. (1989). Cloning, genetic characterization, and nucleotide sequence of the *hemA-prfA* operon of *Salmonella typhimurium*. *J. Bacteriol.* 171 (7): 3948-3960.
- Erskine, P. T., Newbold, R., Roper, J., Coker, A., Warren, M. J., Shoolingin-Jordan, P. M., Wood, S. P. and Cooper, J. B. (1999b). The Schiff base complex of yeast 5-aminolaevulinic acid dehydratase with laevulinic acid. *Prot. Sci.* 8(6): 1250-1256.
- Erskine, P. T., Norton, E., Cooper, J. B., Lambert, R., Lewis, G., Spencer, P., Sarwar, M., Wood, S. P., Warren, M. J. and Shoolingin-Jordan, P. M. (1999a). X-ray structure of 5-aminolevulinic acid dehydratase from *Escherichia coli* complexed with the inhibitor levulinic acid at 2.0Å resolution. *Biochem.* 38 (14): 4266-4276.
- Erskine, P. T., Senior, N., Maignan, S., Cooper, J., Lambert, R., Lewis, G., Spencer, P., Awan, S., Warren, M., Tickle, I. J., Thomas, P., Wood, S. P. and Shoolingin-Jordan, P. M. (1997). Crystallization of 5-aminolaevulinic acid dehydratase from *Escherichia coli* and *Saccharomyces cerevisiae*. *Prot. Sci.* 6 (8): 1774-1776.
- Evans, J., Lefkowitz, J., Lim, C. K. and Billing, B. (1981). Faecal porphyrin abnormalities in a patient with features of Rotor's syndrome. *Gastroenterol.* 81: 1125-1130.
- Faeder, E. J. and Siegel, L. M. (1973). A rapid micromethod for determination of FMN and FAD in mixtures. *Anal. Biochem.* 53: 332-336.
- Falk, J. E. (1964). *Porphyria and metalloporphyria*; Elsevier, Amsterdam.
- Falk, J. E., Dresel, E. I. B. and Rimington, C. (1953). Porphobilinogen as a porphyrin precursor, and interconversion of porphyrins in a tissue system. *Nature* 172: 292.

- Farris, J. (1983). The logical basis of phylogenetic analysis. In: Advances in cladistics, Platnick, I. J. and Funk, V. A eds, Columbia University Press, New York, pp. 1-36.
- Felix, F. and Brouillet, N. (1990). Purification and properties of uroporphyrinogen decarboxylase from *Saccharomyces cerevisiae*. Yeast uroporphyrinogen decarboxylase. Eur. J. Biochem. 188 (2): 393-403.
- Felsenstein, J. (1988). Phylogenies from molecular sequences: inferences and reliability. Ann. Rev. Genet. 22: 521-565.
- Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by author at: joe@genetics.washington.edu.
- Ferreira, G. C. (1994). Mammalian ferrochelatase. Overexpression in *Escherichia coli* as a soluble protein, purification and characterization. J. Biol. Chem. 269 (6): 4396-4400.
- Ferreira, G. C. and Dailey, H. A. (1987). Reconstitution of the two terminal enzymes of the heme biosynthetic pathway into phospholipid vesicles. J. Biol. Chem. 262 (9): 4407-4412.
- Ferreira, G. C. and Dailey, H. A. (1988). Mouse protoporphyrinogen oxidase. Kinetic parameters and demonstration of inhibition by bilirubin. Biochem. J. 250: 597-603.
- Ferreira, G. C., Andrew, T. L., Karr, S. W. and Dailey, H. A. (1988). Organisation of the terminal two enzymes of the heme biosynthetic pathway. Orientation of protoporphyrinogen oxidase and evidence for a membrane complex. J. Biol. Chem. 263 (8): 3835-3839.
- Ferreira, G. C., Franco, R., Lloyd, S. G., Pereira, A.S., Moura, I, Moura, J. J. and Huhn, B. H. (1994). Mammalian ferrochelatase, a new addition to the metalloenzyme family. J. Biol. Chem. 269 (5): 7062-7069.
- Ferreira, G. C., Naeme, P. J. and Dailey, H. A. (1993). Heme biosynthesis in mammalian systems: evidence of a Schiff base linkage between the pyridoxal 5'-phosphate cofactor and a lysine residue in 5-aminolevulinate synthase. Prot. Sci. 2 (11): 1959-1965.
- Ferreira, G. C., Vajapey, U., Hafez, O., Hunter, G. A. and Barber, M. J. (1995). Aminolevulinate synthase: Lysine 313 is not essential for binding the pyridoxal phosphate cofactor but is essential for catalysis. Prot. Sci. 4 (5): 1001-1006.
- Fischer, H. (1915). Über das Koproporphyrin. II. Mitteilung über das Urinporphyrin. Hoppe-Seyler's Z. Physiol. 96: 148-182.
- Fischer, H. and Orth, H. (1934). Die chemie des pyrrols, 3 vols; Johnson Reprint Corp. (Akademische Verlagsgesellschaft MBH, Leipzig, 1934). New York, 1968.
- Fischer, H. and Zerweck, W. (1924). Über uroporphyrinogen heptamethylester und eine neue. Überführung von uro- im koproporphyrin. Hoppe-Seyler's Z. Physiol. Chem. 137: 242-264.
- Folsch, H., Guiard, B., Neupert, W. and Stuart, R. A. (1996). Internal targeting signal of the BCS1 protein: a novel mechanism of import into mitochondria. EMBO. J. 15 (3): 479-487.

- Frank, J., Jugert, F. K., Bretkopf, C., Goerze, G., Merk, G. and Christiano, A. M. (1998b). Recurrent missense mutation in the protoporphyrinogen oxidase gene underlies variegate porphyria. *Am. J. Med. Genet.* 79 (1) : 22-26.
- Frank, J., Jugert, F. K., Kalka, K., Goerz, G., Merk, H. F. and Christiano, A. M. (1998d). Variegate porphyria: identification of a nonsense mutation in the protoporphyrinogen oxidase gene. *J. Invest. Dermatol.* 110 (4): 449-451.
- Frank, J., Lam, H., Zaider, E., Poh-Fitzpatrick, M. and Christiano, A. M. (1998a). Molecular basis of variegate porphyria: a missense mutation in the protoporphyrinogen oxidase gene. *Am. J. Med. Genet.* 35 (3) : 244-247.
- Frank, J., McGrath, J., Lam, H., Graham, R. M., Hawk, J. L. and Christiano, A. M. (1998c). Homozygous variegate porphyria: identification of mutations on both alleles of the protoporphyrinogen oxidase gene in a severely affected proband. *J. Invest. Dermatol.* 110 (4): 452-455.
- Frank, J., Nelson, J., Wang, X., Yang, L., Ahmad, W., Lam, H., Jugert, F. K., Kalka, K., Poh-Fitzpatrick, M. B., Goerz, G., Merk, H. F. and Christiano, A. M. (1999). Erythropoietic protoporphyria: identification of novel mutations in the ferrochelatase gene and comparison of biochemical markers versus molecular analysis as diagnostic strategies. *J. Invest. Med.* 47 (6): 278-284.
- Frank, J., Poh-Fitzpatrick, M. B., King, L. E. Jr and Christiano, A. M. (1998e). The genetic basis of "Scarsdale Gourmet Diet" variegate porphyria: a missense mutation in the protoporphyrinogen oxidase gene. *Arch. Dermatol. Res.* 290 (8): 441-445.
- Frankenberg, N., Erskine, P. T., Cooper, J. B., Shoolingin-Jordan, P. M., Jahn, D. and Heinz, D. W. (1999). High resolution crystal structure of a Mg^{2+} -dependent porphobilinogen synthase. *J. Mol. Biol.* 289 (3): 591-602.
- Frankenberg, N., Kittel, T., Hungerer, C., Romling, U. and Jahn, D. (1998). Cloning, mapping and functional characterization of the *hemB* gene of *Pseudomonas aeruginosa*, which encodes a magnesium-dependent 5-aminolevulinic acid dehydratase. *Molec. Gen. Genet.* 257 (4): 485-489.
- Frear, D.S., Swanson, H. R. and Mansager, E. R. (1983). Acifluorfen metabolism in soybean: diphenyl ether bond cleavage and the formation of homogluthathione, cysteine and glucose conjugates. *Pestic. Biochem. Physiol.* 20: 299-310.
- Frydman, R. B. and Feinstein, G. (1974). Studies on porphobilinogen deaminase and uroporphyrinogen 3 cosynthase from human erythrocytes. *Biochim. Biophys. Acta* 350 (2): 358-373.
- Fuesler, T. P., Castelfranco, P. A. and Wong, Y-S. (1984). *Plant Physiol.* 74: 928-933.
- Furuyama, K., Fujita, H., Nagai, T., Yomogida, K., Munakata, H., Kondo, M., Kimura, A., Kuramoto, A., Hayashi, N. and Yamamoto, M. (1997). Pyridoxine refractory X-linked sideroblastic anemia caused by a point mutation in the erythroid 5-aminolevulinic synthase gene. *Blood* 90 (2): 822-830.
- Furuyama, K., Uno, R., Urabe, A., Hayashi, N., Fujita, H., Kondo, M., Sassa, S. and Yamamoto, M. (1998). R411C mutation of the *ALAS2* gene encodes a pyridoxine-responsive enzyme with low activity. *Br. J. Haematol.* 103 (3): 839-841.

- Gaba, V., Cohen, N., Schaaltiel, Y., Ben-Amotz, A. and Gressel, J. (1988). Light-requiring acifluorfen action in the absence of bulk photosynthetic pigments. *Pestic. Biochem. Physiol.* 31: 1-12.
- Gandolfo, L. D., Macri, A., Biolcati, G., Griso, D., Phung, L., Deybach, J-C., Da Silva, V., Nordmann, Y. and Topi, G. C. (1991). Homozygous variegate porphyria: revision of a diagnostic error. *Brit. J. Med (Lett.)* 124: 211.
- Garey, J. R., Hansen, J. L., Harrison, L. M., Kennedy, J. B. and Kushner, J. P. (1989). A point mutation in the coding region of uroporphyrinogen decarboxylase associated with familial porphyria cutanea tarda. *Blood* 73: 892-895.
- Garey, J. R., Labbe-Bois, R., Chelstowska, A., Rytka, J., Harrison, L., Kushner, J. and Labbe, P. (1992). Uroporphyrinogen decarboxylase from *Saccharomyces cerevisiae*. *HEM12* gene sequence and evidence for two conserved glycines essential for enzymatic activity. *Eur. J. Biochem.* 205 (3): 1011-1016.
- Gatley, S. J. and Sherratt, S. A. (1976). The effects of diphenyleneiodonium and 2,4-dichlorodiphenyleneiodonium on mitochondrial reaction. Relationship of binding of diphenylene[¹²⁵I]iodonium to mitochondria to the extent of inhibition of oxygen uptake. *Biochem. J.* 158 (2): 307-315.
- Ghisla, S. (1980). Fluorescence and optical characteristics of reduced flavins and flavoproteins. *Methods Enzymol.* 66: 360-373.
- Gibbs, P. N. B., Chaudhry, A. G. and Jordan, P. M. (1985). Purification and properties of 5-aminolaevulinate dehydratase from human erythrocytes. *Biochem. J.* 230 (1): 25-34.
- Gibson, K. D., Laver, W. G. and Neuberger, A. (1958). Initial stages in the biosynthesis of porphyrins. 2. The formation of δ -aminolaevulinic acid from glycine and succinyl coenzyme A by particles from chicken erythrocytes. *Biochem. J.* 70: 71-76.
- Gibson, K. D., Neuberger, A. and Scott, J. J. (1955). *Biochem. J.* 61: 618-629.
- Glerum, D. M., Shtanko, A., Tsagoloff, A., Gorman, N. and Sinclair, P. R. (1996). Cloning and identification of *HEM14*, the yeast gene for mitochondrial protoporphyrinogen oxidase. *Yeast* 12: 1421-1425.
- Goldberg, A., Ashenbrucker, M., Cartwright, G. E. and Wintrobe, M. M. (1956). Studies on the biosynthesis of haem *in vitro* by avian erythrocytes. *Blood* 11: 821-833.
- Gomez-Silva, B., Timko, M. P. and Schiff, J. A. (1985). *Planta* 165: 12-22.
- Gora, M., Grzybowska, E., Rytka, J. and Labbe-Bois, R. (1996). Probing the active site residues in *Saccharomyces cerevisiae* ferrochelatase by directed mutagenesis. *In vivo* and *in vitro* analyses. *J. Biol. Chem.* 271 (20): 11810-11816.
- Gough, S. P., Beale, S. I. and Granick, S. (1976). The biosynthesis of delta-aminolevulinic acid from the intact carbon skeleton of glutamic acid in greening barley. *Annals Clin. Res.* 8 (suppl.) 17: 70-73.
- Grandchamp, B. (1998). Acute intermittent porphyria. *Semin. Liv. Dis.* 1998; 18 (1): 17-24.

- Grandchamp, B. and Nordmann, Y. (1977). Decreased lymphocyte coproporphyrinogen III oxidase activity in hereditary coproporphyria. *Biochem. Biophys. Res. Commun.* 74: 1089.
- Grandchamp, B., de Verneuil, H., Beaumont, C., Chretien, S., Walter, O. and Nordmann, Y. (1987). Tissue-specific expression of porphobilinogen deaminase. Two isoenzymes from a single gene. *Eur. J. Biochem.* 162: 105-110.
- Grandchamp, B., Phung, N. and Nordmann, Y. (1978). The mitochondrial localization of coproporphyrinogen III oxidase. *Biochem. J.* 176: 97-102.
- Grandchamp, B., Romeo, P. H., Dubart, A., Raich, N., Rosa, J., Nordmann, Y. and Goossens, M. (1984). Molecular cloning of a cDNA sequence complementary to porphobilinogen deaminase mRNA from rat. *Proc. Natl. Acad. Sci. USA* 81 (16): 5036-5040.
- Grandchamp, B., Weil, D., Nordmann, Y., Van Cong, M. S., de Verneuil, H., Foubert, C. and Gross, M. S. (1983). Assignment of the human coproporphyrinogen oxidase gene to chromosome 9. *Hum. Genet.* 64 (2): 180-183.
- Granick, J. L., Sassa, S. and Kappas, A. (1978). Some biochemical and clinical aspects of lead intoxication. In: *Advances in clinical chemistry*. Bodansky, O. and Latner, A. L. (eds). New York, Academic Press, pp. 287-339.
- Granick, S. (1963). Induction of the synthesis of 5-ALA synthetase in liver parenchymal cells in culture by chemicals inducing acute porphyria. *J. Biol. Chem.* 238: 2247-2250.
- Granick, S. and Mauzerall, D. (1958). Porphyrin biosynthesis in erythrocytes. II. Enzymes converting delta-aminolevulinic acid to coproporphyrinogen. *J. Biol. Chem.* 232: 1119-1140.
- Granick, S. and Sassa, S. (1971). Delta-aminolaevulinic Acid Synthase and the Control of Haem and Chlorophyll Synthesis. In: *Metabolic Regulation*; Vogel, H. J. ed, Academic Press, New York, London, pp. 77-141.
- Gray, C. H. (1952). Isotope studies in porphyria. *Br. Med. Bull.* 8: 229.
- Gregor, A., Kostrewska, E., Tarczynska-Nosal, S. and Strachurska, H. (1994). *Polski Tygodnik Lekarski* 49: 12-13.
- Grinstein, M., Wilkoff, H. M., Pimenta de Mello, R. and Watson, C. J. (1950). Isotopic studies of porphyrin and hemoglobin metabolism. II. The biosynthesis of coproporphyrinogen III in experimental lead poisoning. *J. Biol. Chem.* 182: 723-728.
- Grundy, W. N. and Naylor, G. J. P. (1999). Phylogenetic inference from conserved sites alignments. *J. Exper. Zoology*. 285 (2): 128-139.
- Gunther, H. (1911). Die hamatoporphyrie. *Deutsche Arch. Klin. Med.* 105: 89-146.
- Gunther, H. (1922). Die bedeutung der hamatoporphyrie in der physiologie und pathologie. *Ergebnisse der Allgemeinen Pathol., Pathol. Anat.* 20: 608-764.
- Guo, R., Lim, C. K. and Peters, T. J. (1991). High performance liquid chromatographic assays for protoporphyrinogen oxidase and ferrochelatase in human leucocytes. *J. Chrom.* 566: 383-396.

- Hamilton, J. W., Bement, W. J., Sinclair, P. R., Sinclair, J. F., Alcedo, J. A. and Wetterhahn, K. E. (1991). Heme regulates hepatic 5-aminolevulinate synthase mRNA expression by decreasing mRNA half-life and not by altering its rate of transcription. *Arch. Biochem. Biophys.* 289 (2): 387-392.
- Han, O. and Kim, O. (1996). Effect of hydrophobic environments and reducing agents on the oxidation of protoporphyrinogen IX. *Bull. Korean Chem. Soc.* 17: 659-661.
- Hansson, M. and Al-Karadaghi, S. (1995). Purification, crystallization, and preliminary X-ray analysis of *Bacillus subtilis* ferrochelatase. *Proteins* 23 (4): 607-609.
- Hansson, M. and Hederstedt, L. (1992). Cloning and characterization of the *Bacillus subtilis* *hemEHY* gene cluster, which encodes protoheme IX biosynthetic enzymes. *J. Bacteriol.* 174 (24): 8081-8093.
- Hansson, M. and Hederstedt, L. (1994a). Purification and characterisation of a water-soluble ferrochelatase from *Bacillus subtilis*. *Eur. J. Biochem.* 220 (1): 201-208.
- Hansson, M. and Hederstedt, L. (1994b). *Bacillus subtilis* *hemY* is a peripheral membrane protein essential for protoheme IX synthesis which can oxidize coproporphyrinogen III and protoporphyrinogen IX. *J. Bacteriol.* 176 (19): 5962-5970.
- Hansson, M., Gustafsson, M. C. U., Kannangara, C. G. and Hederstedt, L. (1997). Isolated *Bacillus subtilis* *hemY* has coproporphyrinogen III to coproporphyrin III oxidase activity. *Biochim. et Biophys. Acta* 1340: 97-104.
- Harbin, B. M. and Dailey, H. A. (1985). Orientation of ferrochelatase in bovine liver mitochondria. *Biochem.* 24 (2): 366-370.
- Harigae, H., Furuyama, K., Kimura, A., Neriishi, K., Tahara, N., Kondo, M., Hayashi, N., Yamamoto, M., Sassa, S. and Sasaki, T. (1999a). A novel mutation of the erythroid-specific delta-aminolaevulinate synthase gene in a patient with X-linked sideroblastic anaemia. *Br. J. Haematol.* 106 (1): 175-177.
- Harigae, H., Furuyama, K., Kudo, K., Hayashi, N., Yamamoto, M., Sassa, S. and Sasaki, T. (1999b). A novel mutation of the erythroid-specific gamma-Aminolevulinate synthase gene in a patient with non-inherited pyridoxine-responsive sideroblastic anemia. *Am. J. Hematol.* 62 (2): 112-114.
- Hart, G. J. and Battersby, A. R. (1985). Purification and properties of uroporphyrinogen III synthase (cosynthase) from *Euglena gracilis*. *Biochem. J.* 232 (1): 151-160.
- Hasnain, S. S., Wardell, E. M., Garner, C. D., Schlosser, M. and Beyersmann, D. (1985). Extended-X-ray-absorption-fine-structure investigation of zinc in 5-aminolaevulinate dehydratase. *Biochem. J.* 230: 625-633.
- Hawk, J. L., Magnus, I. A., Parkes, A., Elder, G. H. and Doyle, M. (1978). Deficiency of hepatic coproporphyrinogen oxidase in hereditary coproporphyria. *J. Roy. Soc. Med.* 71: 775-777.
- Hayashi, N., Watanabe, N. and Kikuchi, G. (1983). Inhibition by heme of *in vivo* translocation of chicken liver 5-aminolaevulinate synthase into mitochondria. *Biochem. Biophys. Res. Commun.* 115: 700-706.

- Hayashi, N., Yoda, B. and Kikuchi, G. (1969). Mechanism of allylisopropyl acetamide induced increase of δ -aminolevulinate synthetase in liver mitochondria. IV. Accumulation of the enzyme in the soluble fraction of rat liver. *Arch. Biochem. Biophys.* 131: 83-91.
- Hayashi, N., Yoda, B. and Kikuchi, G. (1970). Differences in molecular sizes of δ -aminolevulinate synthetases in the soluble and mitochondrial fractions of rat liver. *J. Biochem. (Tokyo)* 67: 859-861.
- Hayashi, N., Yoda, B. and Kikuchi, G. (1976). Difference in molecular sizes of ALA synthetases from the soluble and mitochondrial fractions of rat liver. *Cell Tissue Res.* 232:257-264.
- Hebert, G. A., Pelham, P. L. and Pittman, B. (1973). Determination of the optimal ammonium sulphate concentration for the fractionation of rabbit, sheep, horse and goat antisera. *Appl. Microbiol.* 25 (1): 26-36.
- Heinfeld, J. F. and Furuya, F. R. (1995). Silver enhancement of Nanogold and undecagold. In: *Immunogold silver staining – Principles, Methods and Applications*, Hayat, M. A. eds, Academic Press, San Diego.
- Held, J. L., Sassa, S., Kappas, L., et al (1989). Erythrocyte uroporphyrinogen decarboxylase activity in porphyria cutanea tarda: a study of 40 consecutive patients. *J. Invest. Dermatol.* 93: 332-334.
- Hift, R. J., Meissner, P. N. and Meissner, D. M. (1993). *Porphyria, a guide for people with porphyria and their doctors* MRC/UCT Liver Research Centre, Cape Town.
- Hift, R. J., Meissner, P. N., Corrigall, A. V., Ziman, M. R., Petersen, L. A., Meissner, D. M., Davidson, B. P., Sutherland, J., Dailey, H. A. and Kirsch, R. E. (1997). Variegate porphyria in South Africa, 1688-1996 - new developments in an old disease. *S. Afr. Med. J.* 87 (6): 718-727.
- Higuchi, M. and Bogorad, L. (1975). The purification and properties of uroporphyrinogen I synthetase and uroporphyrinogen III cosynthetase. Interaction between the enzymes. *Ann. N. Y. Acad. Sci.* 244: 401-408.
- Hindmarsh, J. T. (1993). Variable phenotypic expression of genotypic abnormalities in the porphyrias. *Clin. Chim. Acta* 217: 29-38.
- Hippler, B., Homuth, G., Hoffmann, T., Hungerer, C., Schumann, W. and Jahn, D. (1997). Characterization of *Bacillus subtilis hemN*. *J. Bacteriol.* 179 (22): 7181-7185.
- Hoffmann, T., Troup, B., Szabo, A., Hungerer, C. and Jahn, D. (1995). The anaerobic life of *Bacillus subtilis*. Cloning and characterization of the genes encoding the respiratory nitrate reductase system. *FEMS Microbiol. Lett.* 131: 219-225.
- Homuth, G., Heinemann, M., Zuber, U. and Schumann, W. (1995). The genes *lapA* and *hemN* form a bicistronic operon in *Bacillus subtilis*. *Microbiol.* 142: 1641-1649.
- Hooper, J. K., Kahn, A., Ash, D. E., Gough, S. P. and Kannangara, C. G. (1988). Biosynthesis of delta-aminolevulinate in greening barley leaves. IX. Structure of the substrate, mode of gabaculine inhibition, and the catalytic mechanism of glutamate-1-semialdehyde aminotransferase. *Carlsberg Res. Comm.* 53 (1): 11-25.

- Hopf, F. R. and Whiten, D. G. (1978). In: The Porphyrins Vol. 2, Dolphin, D. ed., Academic Press, New York, pp. 161-195.
- Hoppe-Seyler, F. (1871). Das Hamatin. *Tubinger Med-Chem. Untersuchungen* 4: 523-533.
- Hoppe-Seyler, F. (1879). Über das Chlorophyll der Pflanzen. *Hoppe-Seyler's Z Physiologie Chem.* 4: 193-203.
- Horrisberger, M. and Rosset, J. (1977). Colloidal gold, a useful marker for transmission and scanning electron microscopy. *J. Histochem. Cytochem.* 25: 295-305.
- Houen, G., Gough, S. P. and Kannangara, C. G. (1984). 5-ALA synthesis in greening barley leaves. V. The structure of glutamate-1-semialdehyde. *Carlsberg Res. Comm.* 52: 567-572.
- Huang, L. and Castelfranco, P. A. (1988). *Plant Sci.* 54: 185-192.
- Ichinose, K., Che, F-S., Kimura, Y., Matsunobu, A., Sato, F. and Yoshida, S. (1995). Selection and characterisation of protoporphyrinogen oxidase-inhibiting herbicide (S-23142) resistant photomixotrophic cultured cells of *Nicotiana tabacum*. *J. Plant Physiol.* 146: 693-698.
- Ishida, N., Fujita, N., Noguchi, T., et al, (1990). Message amplification phenotyping of an inherited δ -aminolevulinate dehydratase deficiency in a family with acute hepatic porphyria. *Biochem. Biophys. Res. Commun.* 172: 237-242.
- IUPAC-IUB Joint Commission on Biochemical Nomenclature (1980): Nomenclature of Tetrapyrroles; Recommendations, 1978. *Eur. J. Biochem.* 108: 1-30.
- Jackson, A. H., Games, D. E., Couch, P., Jackson, J. R., Belcher, R. B. and Smith, S. G. (1974). Conversion of coproporphyrinogen III to protoporphyrin IX. *Enzyme* 17: 81-87.
- Jackson, A. H., Sancovich, A. H., Ferramolo, A. M., Evans, N., Games, D. E., Mattin, S. A., Elder, G. H. and Smith, S. G. (1976). Macrocyclic intermediates in the biosynthesis of the porphyrins. *Philos Trans. Roy. Soc. Lond. B* 273: 191-206.
- Jacobs, J. M. and Jacobs, N. J. (1977b). The late steps of anaerobic heme biosynthesis in *Escherichia coli*: role for quinones in protoporphyrinogen oxidation. *Biochem. Biophys. Res. Commun.* 78 (1): 429-433.
- Jacobs, J. M. and Jacobs, N. J. (1984a). Protoporphyrinogen oxidation, an enzymatic step in heme and chlorophyll synthesis: Partial characterization of the reaction in plant organelles and comparison with mammalian and bacterial systems. *Arch. Biochem. Biophys.* 229 (1): 312-319.
- Jacobs, J. M. and Jacobs, N. J. (1984b). Effect of unsaturated fatty acids on protoporphyrinogen oxidation, a step in heme and chlorophyll synthesis in plant organelles. *Biochem. Biophys. Res. Commun.* 123 (3): 1157-1164.
- Jacobs, J. M. and Jacobs, N. J. (1987). Oxidation of protoporphyrinogen to protoporphyrin, a step in chlorophyll and haem biosynthesis. Purification and partial characterization of the enzyme from barley organelles. *Biochem. J.* 244: 219-224.
- Jacobs, J. M. and Jacobs, N. J. (1995). Terminal enzymes of heme biosynthesis in the plant plasma membrane. *Arch. Biochem. Biophys.* 323 (2): 274-278.

- Jacobs, J. M., Jacobs, N. J., Borotz, S. E. and Guerinot, M. L. (1990). Effects of photobleaching herbicide, acifluorfenmethyl, on protoporphyrinogen oxidation in barley organelles, soybean root mitochondria, soybean root nodules, and bacteria. *Arch. Biochem. Biophys.* 280 (2): 369-375.
- Jacobs, J. M., Jacobs, N. J. and Duke, S. O. (1996). Protoporphyrinogen destruction by plant extracts and correlation with tolerance to protoporphyrinogen oxidase inhibiting herbicides. *Pestic. Biochem. Physiol.* 55: 77-83.
- Jacobs, J. M., Jacobs, N. J., Sherman, T. D. and Duke, S. O. (1991). Effect of diphenyl ether herbicides on oxidation of protoporphyrinogen to protoporphyrin in organellar and plasma membrane enriched fraction of barley. *Plant Physiol.* 97: 197-203.
- Jacobs, J. M., Sinclair, P. R., Gorman, N., Jacobs, N. J., Sinclair, J. F., Bement, W. J. and Walton, H. (1992). Effects of diphenyl ether herbicides on porphyrin accumulation by cultured hepatocytes. *J. Biochem. Toxicol.* 7 (2): 87-95.
- Jacobs, N. J. and Jacobs, J. M. (1975). Fumarate as an alternative electron acceptor for the late steps of anaerobic heme synthesis in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 65 (1): 435-441.
- Jacobs, N. J. and Jacobs, J. M. (1976). Nitrate, fumarate and oxygen as electron acceptors for a late step in microbial heme synthesis. *Biochim. et Biophys. Acta* 449: 1-9.
- Jacobs, N. J. and Jacobs, J. M. (1977a). Evidence for involvement of the electron transport system at a late step of microbial heme synthesis. *Biochim et Biophys. Acta* 459: 141-144.
- Jacobs, N. J. and Jacobs, J. M. (1981). Protoporphyrinogen oxidation in *Rhodospseudomonas spheroides*, a step in heme and bacteriochlorophyll synthesis. *Arch. Biochem. Biophys.* 211 (1): 305-311.
- Jacobs, N. J., Borotz, S. E. and Jacobs, J. M. (1989). Characteristics of purified protoporphyrinogen oxidase from barley. *Biochem. Biophys. Res. Commun.* 161 (2): 790-796.
- Jacobs, N. J., Jacobs, J. M. and De Maggio, A. (1982). Protoporphyrinogen oxidation in chloroplasts and plant mitochondria, a step in heme and chlorophyll synthesis. *Arch. Biochem. Biophys.* 218: 233-239.
- Jenkins, T. (1990). Medical genetics in South Africa. *J. Med. Genet.* 27: 760-769.
- Jenkins, T. (1996). The South African malady. *Nat. Genet.* 13: 7-9.
- Jenkins, T. (1997). The molecular basis of South African genetic porphyria established at last. *S. Afr. Med. J.* 87 (6): 733-735.
- Johston, D. J., Droz, E., Rochaix, J-D. and Malno, P. (1998). Cloning and characterisation of potato cDNAs involved in tetrapyrrole biosynthesis (Accession numbers: ferrochelatase AJ005802, chloroplast protoporphyrinogen IX oxidase AJ225107, mitochondrial protoporphyrinogen IX oxidase AJ225108). *Plant Physiol.* 118: 330.
- Jones, M. S. and Jones, O. T. G. (1969). The structural organisation of heme synthesis in the rat liver mitochondria. *Biochem. J.* 113: 507.
- Jones, O. T. G. (1976). Chlorophyll biosynthesis. *Phil. Trans. Soc. Lond. (Biol.)* 273: 207-225.

- Jones, R. M. and Jordan, P. M. (1993). Purification and properties of uroporphyrinogen decarboxylase from *Rhodobacter sphaeroides*. *Biochem. J.* 293 (3): 703-712.
- Jordan, P. M. (1990). Biosynthesis of 5-aminolaevulinic acid and its transformation into coproporphyrinogen in animals and bacteria. In: *Biosynthesis of Heme and Chlorophylls*; Dailey, H. A. ed., McGraw Hill publishing Co. New York, pp. 55-122.
- Jordan, P. M. (1994). The biosynthesis of uroporphyrinogen-III: mechanism of action of porphobilinogen deaminase. *Ciba Foundation Symp.* 180: 70-89; discussion 89-96.
- Jordan, P. M. and Seehra, J. S. (1980). Mechanism of action of δ -aminolevulinic acid dehydratase. Stepwise order of addition of the two molecules of δ -aminolevulinic acid in the enzyme during synthesis of porphobilinogen. *J. Chem. Soc. Chem. Commun.* 240: 242.
- Jordan, P. M. and Seehra, J. S. (1986). Purification of porphobilinogen synthase from bovine liver. *Methods Enzymol.* 123: 427-434.
- Jordan, P. M. and Shemin, D. (1972). Delta-aminolevulinic Acid Synthetase. In: *The Enzymes*, 3rd ed., Vol. 7; Bover, P. D. ed., Academic Press, New York, 339-356.
- Jordan, P. M. and Shemin, D. (1973). Purification of uroporphyrinogen I synthetase from *R. sphaeroides*. *J. Biol. Chem.* 243: 1019-1024.
- Jordan, P. M. and Warren, M. J. (1987). Evidence for a dipyrromethane cofactor at the catalytic site of *Escherichia coli* porphobilinogen deaminase. *FEBS Lett.* 225: 87-92.
- Jordan, P. M., Mbeje, I. A. B., Alwan, A. F. and Thomas, S. D. (1987). Nucleotide sequence of *hemD*, the second gene in the *hem* operon of *Escherichia coli* K12. *Nucl. Acids Res.* 15 (24): 10583.
- Jordan, P. M., Thomas, S. D. and Warren, M. J. (1988). Purification, crystallization and properties of porphobilinogen deaminase from a recombinant strain of *Escherichia coli* K12. *Biochem. J.* 254: 427-435.
- Jordan, P. M., Warren, M. J., Mgbeje, B. I., Wood, S. P., Cooper, J. B., Louie, G., Brownlie, P., Lambert, R. and Blundell, T. L. (1992). Crystallization and preliminary X-ray investigation of *Escherichia coli* porphobilinogen deaminase. *J. Mol. Biol.* 224 (1): 269-271.
- Juknat, A. A., Seubert, A., Seubert, S. and Ippen, H. (1989). Studies on uroporphyrinogen decarboxylase of etiolated *Euglena gracilis* Z.. *Eur. J. Biochem.* 179 (2): 423-428.
- Kaczor, C. M., Smith, M. W., Sangwan, I. and O'Brian, M. R. (1994). Plant delta-aminolevulinic acid dehydratase. Expression in soybean root nodules and evidence for a bacterial lineage of the ALAD gene. *Plant Physiol.* 104 (4): 1411-1417.
- Kannangara, C. G., Gough, S. P., Bruyant, P., Hooper, J. K., Kahn, A. and von Wettstein, D. (1988). tRNA(Glu) as a cofactor in delta-aminolevulinate biosynthesis: steps that regulate chlorophyll synthesis. *Trends Biochem. Sci.* 13 (4): 139-143.

- Kappas, A., Sassa, S., Galbraith, R. A. and Nordmann, Y. (1989). The Porphyrrias. In: *The Metabolic Basis of Inherited Disease*, Scriver, C. L., Beaudet, A. L., Sly, W. S. and Valle, D. eds, McGraw-Hill, New York, pp. 1305-1365.
- Kappas, A., Sassa, S., Galbraith, R. A., et al, (1995). The porphyrias. In: *Metabolic and molecular basis of inherited disease*, Scriver, S., Beaudet, A. L. and Sly, W. S. eds, 7th ed.. New York, McGraw-Hill, pp. 2103-2159.
- Kasher, M. S., Walkuchik, M., Cook, J. M. and Smith, M. C. (1993). One-step purification of human papillomavirus type 16 E7 oncoprotein and its binding to the retinoblastoma gene product. *Biotechniques* 14(4): 630-641.
- Kataoka, M., Sato, R., and Oshio, H. (1990). Isolation and partial characterization of mutant *Chlamydomonas reinhardtii* protoporphyrinogen oxidase resistant to herbicide S-23142. *J. Pestic. Sci.* 15: 449-451.
- Kauppinen, R. and Mustajoki, P. (1992). Prognosis of acute porphyria: Occurrence of acute attacks, precipitating factors, and associated diseases. *Medicine* 71 (1): 1-13.
- Kauppinen, R., Mustajoki, S., Pihlaja, H., Peltonen, N. and Mustajoki, P. (1995). Acute intermittent porphyria in Finland: 19 mutations in the uroporphobilinogen deaminase gene. *Hum. Mol. Genet.* 4: 215-222.
- Kauppinen, R., Timonen, K., Taketani, S. and Mustajoki, P. (1996). Homozygous variegate porphyria: ten years follow-up and characterisation of a molecular defect. *Hepatology* 23: 1-90.
- Kawanishi, S., Seki, Y. and Sano, S. (1983). Uroporphyrinogen decarboxylase - purification, properties and inhibition by polychlorinated biphenyl isomers. *J. Biol. Chem.* 258: 4285-4292.
- Kaya, A. H., Plewinska, M., Wong, D. M., Desnick, R. J. and Wetmur, J. G. (1994). Human δ -aminolevulinic acid dehydratase (ALAD) gene: structure and alternative splicing of the erythroid and housekeeping mRNAs. *Genomics* 19: 242-248.
- Kiel, J. A., Ten Berge, A. M. and Venema, G. (1992). Nucleotide sequence of the *Synechococcus* sp. PCC7942 *hemE* gene encoding the homologue of mammalian uroporphyrinogen decarboxylase. *DNA Sequence* 2 (6): 415-418.
- Kikuchi, G., Kumar, A., Talmage, P. and Shemin, D. (1958a). The enzymatic synthesis of 5-ALA. *J. Biol. Chem.* 233: 1214-1219.
- Kikuchi, G., Shemin, D. and Bachmann, B. J. (1958b). The enzymatic synthesis of 5-aminolevulinic acid. *Biochem. Biophys. Acta* 28: 219-220.
- Kikuchi, G. and Hayashi, N. (1981). Regulation by heme of synthesis and intracellular translocation of 5-aminolevulinic acid synthase in the liver. *Mol. Cell Biochem.* 37: 27-41.
- Kirsch, R. E., Fleischner, G., Kamisaka, J. and Arias, I. M. (1975). *J. Clin. Invest.* 55: 1009-1019.
- Kirsch, R. E., Meissner, P. N. and Hift, R. J. (1998). Variegate porphyria. *Semin. Liver Dis.* 18 (1): 33-41.

- Klatskin, G. and Bloomer, J. R. (1974). Birefringence of hepatic pigment deposits in erythropoietic protoporphyria. Specificity and sensitivity of polarization microscopy in the identification of porphyrin deposits. *Gastroenterol.* 67: 294-302.
- Klausner, R. D., Rounault, T. A., Harford et al (1993). Regulating the fate of mRNA: The control of cellular iron metabolism. *Cell* 72: 19-28.
- Klemm, D. J. and Barton, L. L. (1985). Oxidation of protoporphyrinogen in the obligate anaerobe *Desulfovibrio gigas*. *J. Bacteriol.* 164 (1): 316-320.
- Klemm, D. J. and Barton, L. L. (1987). Purification and properties of protoporphyrinogen oxidase from an anaerobic bacterium, *Desulfovibrio gigas*. *J. Bacteriol.* 169 (11): 5209-5215.
- Klemm, D. J. and Barton, L. L. (1989). Protoporphyrinogen oxidation coupled to nitrate reduction with membranes from *Desulfovibrio gigas*. *FEMS Microbiol. Lett.* 61: 61-64.
- Kohashi, M., Clement, R. P., Tse, J. and Piper, W. N. (1984). Rat hepatic uroporphyrinogen III cosynthase. Purification and evidence for a bound folate coenzyme participating in the biosynthesis of uroporphyrinogen III. *Biochem. J.* 220: 755-765.
- Kohno, H., Furukawa, T., Tokunaga, R., Taketani, S. and Yoshinaga, T. (1996). Mouse coproporphyrinogen oxidase is a copper-containing enzyme: Expression in *Escherichia coli* and site-directed mutagenesis. *Biochem. et Biophys. Acta* 1292 (1): 156-162.
- Kohno, H., Furukawa, T., Yoshinaga, T., Tokunaga, R. and Taketani, S. (1993). Coproporphyrinogen oxidase. Purification, molecular cloning, and induction of mRNA during erythroid differentiation. *J. Biol. Chem.* 268 (28): 21359-21363.
- Kolarov, J., Nelson, B. D. and Kuzela, S. (1983). Rat liver protoporphyrinogen IX oxidase: site of synthesis and factors influencing its activity. *Biochem. Biophys. Res. Commun.* 116 (2): 383-387.
- Komives, T. and Gullner, G. (1994). Mechanisms of plant tolerance to photodynamic herbicides. *Amer. Chem. Soc. Symp. Ser.* 559: 177-190.
- Korda, V., Deybach, J.-C., Martasek, P., Zeman, J., Da Silva, V., Nordmann, Y., Houstkova, H., Rubin, A. and Holub, J. (1984). Homozygous variegate porphyria. *Lancet (Lett.)* 1: 851.
- Koziol, J. (1971). Fluorometric analysis of riboflavin and its coenzymes. *Methods Enzymol.* 18B: 253-285.
- Kramer, S. (1980). Porphyria Variegata. *Clin. Haematol.* 9: 303-322.
- Krijt, J., Stranska, P., Maruna, P., Vokurka, M. and Sanitrak, J. (1997). Herbicide-induced experimental variegate porphyria in mice: tissue porphyrinogen accumulation and response to porphyrogenic drugs. *Can J. Physiol. Pharmacol.* 75: 1181-1187.
- Kushner, J. P., Barbutto, A. J. and Lee, G. R. (1976). An inherited enzymatic defect in porphyria cutanea tarda: decreased uroporphyrinogen decarboxylase activity. *J. Clin. Invest.* 58: 1089-1097.
- Kuster, W. (1912). Beitrage zur kenntnis das bilirubins and hamins. *Hoppe-Seyler's Z. Physiol. Chem.* 82: 463-483.

- Labbe-Bois, R. (1990). The ferrochelatase from *Saccharomyces cerevisiae*. Sequence, disruption and expression of its structural gene *HEM15*. J. Biol. Chem. 265 (13): 7278-7283.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature (London) 227: 680-685.
- Laidlaw, P. P. (1904). Some observations on blood pigments. J. Physiol. 31: 464-472.
- Lam, H., Dragan, L., Tsou, H. C., Merk, H., Peacocke, M., Goerze, G., Sassa, S., Poh-Fitzpatrick, M., Bickers, B. R. and Christiano, A. M. (1997). Molecular basis of variegate porphyria: a *de novo* insertion in the protoporphyrinogen oxidase gene. Hum. Genet. 99: 126-129.
- Lambert, R., Brownlie, P. D., Woodcock, S. C., Louie, G. V., Cooper, J. C., Warren, M. J., Jordan, P. M., Blundell, T. L. and Wood, S. P. (1994). Structural studies on porphobilinogen deaminase. Ciba Foundation Symp. 180: 97-104; discussion 105-110.
- Lamon, J. M., Frykholm, B. G., and Tschudy, D. P. (1979). Family evaluation in acute intermittent porphyria using red cell uroporphyrinogen I synthase. J. Med. Genet. 16: 134-139.
- Laterriere, M., d'Estaintot, B. L., Dautant, A., Precigoux, G., Hombrados, I. and de Verneuil, H. (1997). Expression, purification, crystallization and preliminary X-ray diffraction analysis of human uroporphyrinogen decarboxylase. Acta Crystallogr. D. Biol. Crystallogr. 54: 476-478.
- Lathrop, J. T. and Timko, M. P. (1993). Regulation by heme of mitochondrial protein transport through a conserved amino acid motif. Science 259: 522-525.
- Le Guen, L., Santos, R. and Camadro, J-M. (1999). Functional analysis of the *hemK* gene product involvement in protoporphyrinogen oxidase activity in yeast. FEMS Microbiol. Lett. 173 (10): 175-182.
- Le Noble, C. (1887). Arch. Ger Physiol. 40: 501.
- Lecanu, L. R. (1837). Etudes chimiques sur le sang humain.
- Lee, H. J. and Duke, S. O. (1994). Protoporphyrinogen-IX oxidizing activities involved in the mode of action of peroxidizing herbicides. J. Agric. Food Chem. 42: 2610-2618.
- Lee, H. J., Duke, M. V. and Duke, S. O. (1993). Cellular localization of protoporphyrinogen oxidizing activities of etiolated barley (*Hordeum vulgare* L.) leaves. Plant Physiol. 102: 881-889.
- Lee, H. J., Lee, S. B., Chung, J. S., Han, S. U., Han, O., Guh, J. O., Jeon, J. S., An, G. and Back, K. (2000). Transgenic rice plants expressing a *Bacillus subtilis* protoporphyrinogen oxidase gene are resistant to diphenyl ether herbicide oxyfluorfen. Plant Physiol. 41(6): 743-749.
- Lee, J. S. and Anvret, M. (1991). Identification of the most common mutation within the porphobilinogen deaminase gene in Swedish patients with acute intermittent porphyria. Proc. Natl. Acad. Sci. (USA) 88: 10912-10915.
- Leeper, F. J. (1994). The evidence of a spirocyclic intermediate in the formation of uroporphyrinogen III by cosynthase. Ciba Foundation Symposium 180: 111-123.
- Lemberg, R. and Legge, J. W. (1949). Haematin Compounds and Bile Pigments, Interscience, New York.

- Lermontova, I. and Grimm, B. (2000). Overexpression of plastidic protoporphyrinogen IX oxidase leads to resistance to the diphenyl-ether herbicide acifluorfen. *Plant Physiol.* 122: 75-83.
- Lermontova, I., Kruse, E., Mock, H-P. and Grimm, B. (1997). Cloning and characterization of a plastidal and a mitochondrial isoform of tobacco protoporphyrinogen IX oxidase. *Proc. Natl. Acad. Sci. USA* 94: 8895-8900.
- Li, J. M., Russell, C. S. and Cosloy, S. D. (1989). Cloning and structure of the *hemA* gene of *Escherichia coli* K-12. *Gene* 82 (2): 209-217.
- Liedgens, W., Lutz, C. and Schneider, H. A. W. (1983). Molecular properties of 5-aminolevulinic acid dehydratase from *Spinacia oleracea*. *Eur. J. Biochem.* 135 (1): 75-79.
- Lill, R., Nargang, L. E. and Neupert, W. (1996). Biogenesis of mitochondrial proteins. *Curr. Opin. Cell Biol.* 8 (4): 505-512.
- Llambias, E. B. G. and Battle, A. M. del C. (1970). Porphyrin biosynthesis in soybean callus. IX. The porphobilinogen deaminase-uroporphyrinogen III cosynthetase kinetic studies. *Biochim. Biophys. Acta* 220 (3): 552-559.
- Logan, G. M., Weiner, M. K., Ellefson, M., Pierach, C. A. and Bloomer, J. R. (1991). Bile porphyrin analysis in the evaluation of variegate porphyria. *New Engl. J. Med.* 324: 1408-1411.
- Long, C., Smyth, S. J., Woolf, J., Murphy, G. M., Finlay, A. Y., Newcombe, R. G. and Elder, G. H. (1993). Detection of latent porphyria by fluorescence emission spectroscopy of plasma. *Br. J. Dermatol.* 129: 9-13.
- Louie, G. V., Brownlie, P. D., Lambert, R., Cooper, J. B., Blundell, T. L., Wood, S. P., Warren, M. J., Woodcock, S. C. and Jordan, P. M. (1992). Structure of porphobilinogen deaminase reveals a flexible multidomain polymerase with a single catalytic site. *Nature* 359 (6390): 33-39.
- Louie, G. V., Brownlie, P. D., Lambert, R., Cooper, J. V. and Blundell, T. L. (1996). The three dimensional structure of *Escherichia coli* porphobilinogen deaminase at 1.76 Å resolution. *Proteins* 25 (1): 48-78.
- Luke, A. M. and Shoolingin-Jordan, P. M. (1998). Dipyrrromethane cofactor assembly in porphobilinogen deaminase. *Biochem. Soc. Trans.* 26: S285-S286.
- Luo, J. and Lim, C. K. (1993). Order of uroporphyrinogen III decarboxylation on incubation of porphobilinogen and uroporphyrinogen III with erythrocyte uroporphyrinogen decarboxylase. *Biochem. J.* 289: 529-532.
- MacDonald, D. M., Germain, D. and Perrot, H. (1981). The histopathology and ultrastructure of liver disease in erythropoietic protoporphyria. *Br J. Dermatol.* 104: 7-17.
- Macmunn, C. A. (1880). *Proc. Royal Soc. Brit.* 31: 206.
- Macmunn, C. A. (1885). *J. Physiol.* 6: 22.
- Madsen, O., Sandal, L., Sandal, N. N. and Marcker, K. A. (1993). A soybean coproporphyrinogen oxidase gene is highly expressed in root nodules. *Plant Molec. Biol.* 23 (1): 35-43.

- Maguire, D. J., Day, A. R., Borthwick, I. A., Srivastava, G., Wigley, P. L., May, B. K. and Elliott, W. H. (1986). Nucleotide sequence of the chicken 5-aminolevulinate synthase gene. *Nucl. Acids Res.* 14 (3): 1379-1391.
- Mailer, K., Poulson, R., Dolphin, D. and Hamilton, A.D. (1980). Ferrochelatase: isolation and purification via affinity chromatography. *Biochem. Biophys. Res. Commun.* 96: 777-784.
- Maizel, J. V. (1971). *Methods Virol.* 5: 179-246.
- Majumdar, D., Avissar, V. J., Wyche, J. H. and Beale, S. I. (1991). Structure and expression of the *Chlorobium vibrioforme hemA* gene. *Arch. Microbiol.* 156 (4): 281-289.
- Marks, G. S. (1969). *Heme and Chlorophyll: Chemical, Biochemical, and Medical Aspects.* Van Nostrand-Rheinholds, Princeton, New Jersey.
- Martasek, P., Kordac, V. and Jirsa, M. (1983). Variegate porphyria and porphyria cutanea tarda. *Arch. Dermatol.* 119: 537-538.
- Martasek, P. (1998). Hereditary coproporphyria. *Semin. Liv. Dis.* 18 (1): 25-32.
- Martasek, P., Camadro, J-M., Delfrau-Larue, M. H., Dumas, J. B., Mantagne, J. J., de Verneuil, H., Labbe, P. and Grandchamp, B. (1994). Molecular cloning, sequencing and functional expression of a cDNA encoding human coproporphyrinogen oxidase. *Proc. Natl. Acad. Sci. USA* 91 (8): 3024-3028.
- Martasek, P., Camadro, J-M., Raman, C. S., Lecomte, S. C., Le Caer, J. P., Demeler, B., Grandchamp, B. and Labbe, P. (1997). Human coproporphyrinogen oxidase. Biochemical characterization of recombinant, normal and R231W mutated enzymes expressed in *Escherichia coli* as soluble, catalytically active homodimers. *Cell Mol. Biol.* 43: 47-58.
- Matilla, A. and Molland, E. A. (1974). A light and electron microscopic study of the liver in case of erythropoietic protoporphyria and in Griseofulvin-induced porphyria in mice. *J. Clin. Pathol.* 27: 698-709.
- Matringe, M. and Scalla, R. (1987). Photoreceptors and electron flow involvement in the achlorophyllous soybean cells. *Pestic. Biochem. Physiol.* 27: 267-274.
- Matringe, M. and Scalla, R. (1988). Effects of acifluorfen-methyl on cucumber cotyledons: protoporphyrin accumulation. *Pestic. Biochem. Physiol.* 32: 164-168.
- Matringe, M., Camadro, J-M., Block, M. A., Joyard, J., Scalla, R., Labbe, P. and Douce, R. (1992a). Localization within chloroplasts of protoporphyrinogen oxidase, the target enzyme for diphenyl ether-like herbicides. *J. Biol. Chem.* 267 (7): 4646-4651.
- Matringe, M., Camadro, J-M., Labbe, P. and Scalla, R. (1989a). Protoporphyrinogen oxidase as a molecular target for diphenyl ether herbicides. *Biochem. J.* 260: 231-235.
- Matringe, M., Camadro, J-M., Labbe, P. and Scalla, R. (1989b). Protoporphyrinogen oxidase inhibition by three peroxidizing herbicides: oxadiazon, LS 82-556 and M&B 39279. *FEBS Lett.* 245 (1, 2): 35-38.

- Matringe, M., Mornet, R. and Scalla, R. (1992b). Characterization of [³H]acifluorfen binding to pea etioplasts, and evidence that protoporphyrinogen oxidase specifically binds acifluorfen. *Eur. J. Biochem.* 209 (3): 861-868.
- Matsumoto, H., Lee, J. J. and Ishizuka, K. (1994). Variation in crop response to protoporphyrinogen oxidase inhibitors. *Amer. Chem. Soc. Symp. Ser.* 559: 120-132.
- Matters, G. L. and Beale, S. I. (1995). Structure and expression of the *Chlamydomonas reinhardtii* ALAD gene encoding the chlorophyll biosynthetic enzyme, delta-aminolevulinic acid dehydratase (porphobilinogen synthase). *Plant Molec. Biol.* 27 (3): 607-617.
- Mauzerall, D. and Granick, S. (1958). *J. Biol. Chem.* 232: 1141-1162.
- May, B. K., Dogra, S. C., Sadlon, T. J. et al (1995). *Prog. Nucl. Acid Res. Mol. Biol.* 51: 1-51.
- McClung, C. R., Somerville, J. E., Gueriot, M. L. and Chelm, B. K. (1987). Structure of the *Bradyrhizobium japonicum* gene *hema* encoding 5-aminolevulinic acid synthase. *Gene* 54 (1): 133-139.
- McColl, K. E., Thompson, G. G., el Omar, E., Moore, M. R. and Goldberg, A. (1987). Porphyrin metabolism and haem biosynthesis in Gilbert's syndrome. *Gut* 28 (2): 125-130.
- McDonagh, A. F. and Bissell, D. M. (1998). Porphyria and porphyrinology - The past fifteen years. *Semin. Liv. Dis.* 18 (1): 3-15.
- McKay, R., Druyan, R., Getz, G. S and Rabinowitz, M. (1969). Intramitochondrial localization of δ-aminolevulinic acid synthetase and ferrochelatase in rat liver. *Biochem. J.* 114: 455-461.
- McLellan, T., Pryor, M. A., Kushner, J. P., Eddy, R. L. and Shows, T. B. (1985). Assignment of uroporphyrinogen decarboxylase (UROD) to the pter----p21 region of human chromosome 1. *Cytogenet. Cell Genet.* 39 (3): 224-227.
- McManus, J. F., Begley, C. G., Sassa, S. and Ratnaik, S. (1996). Five new mutations in the uroporphyrinogen decarboxylase gene identified in families with cutaneous porphyria. *Blood* 88: 3586-3600.
- Medlock, A. E. and Dailey, H. A. (1996). Human coproporphyrinogen oxidase is not a metalloprotein. *J. Biol. Chem.* 271 (51): 32507-32510.
- Meguro, K., Fujita, H., Ishida, N. et al (1994). Molecular defects of uroporphyrinogen decarboxylase in a patient with mild hepatoerythropoietic porphyria. *J. Invest. Dermatol.* 102: 681-685.
- Meissner, P. and Hift, R. (1991). Porphyria. In: *Liver Update – a practical guide to the diagnosis and management of liver disease*; Kirsch, R., Robson, S. and Meissner, P. eds., University of Cape Town Printing Department, pp. 199-228.
- Meissner, P. N. (1990). *Enzyme Studies in Variegate Porphyria*. PhD Thesis, University of Cape Town.
- Meissner, P. N., Adams, P. and Kirsch, R. E. (1993). Allosteric inhibition of human lymphoblast and purified porphobilinogen deaminase by protoporphyrinogen and coproporphyrinogen. A possible mechanism for the acute attack of variegate porphyria. *J. Clin. Invest.* 91 (4): 1436-1444.

- Meissner, P. N., Dailey, T. A., Hift, R. J., Ziman, M., Corrigan, A. V., Roberts, A. G., Meissner, D. M., Kirsch, R. E. and Dailey, H. A. (1996). A R59W mutation in human protoporphyrinogen oxidase results in decreased enzyme activity and is prevalent in South Africans with variegate porphyria. *Nat. Genet.* 13: 95-97.
- Meissner, P. N., Day, R. S., Moore, M. R., Disler, P. B. and Harley, E. (1986). Protoporphyrinogen oxidase and porphobilinogen deaminase in variegate porphyria. *Eur. J. Clin. Invest.* 16: 257-261.
- Meissner, P. N., Meissner, D. M., Sturrock, E. D., Davidson, B. P. and Kirsch, R. E. (1987). Porphyria - The UCT experience. *S. Afr. Med. J.* 72: 755-761.
- Melefors, O., Goossen, B., Johansson, R. S., et al (1993). Translational control of 5-aminolevulinate synthase mRNA by iron-responsive elements in erythroid cells. *J. Biol. Chem.* 268: 5974-5978.
- Meyer, U. A. (1973). Intermittent acute porphyria: Clinical and biochemical studies of disordered heme biosynthesis. *Enzyme* 16: 334-342.
- Mignotte, V., Wall, L., de Boer, E., et al (1989). Two tissue-specific factors bind the erythroid promoter of human porphobilinogen deaminase gene. *Nucl. Acid Res.* 17: 37-54.
- Miyagi, K. (1970). Deficiency of hepatic porphobilinogen deaminase in acute intermittent porphyria. *J. Kyushu Haematol. Soc.* 20: 190-203.
- Mock, H. P., Trainotti, L., Kruse, E. and Grimm, B. (1995). Isolation, sequencing and expression of cDNA sequences encoding uroporphyrinogen decarboxylase from tobacco and barley. *Plant Molec. Biol.* 28 (2): 245-256.
- Moore, M. R., McColl, M. E. L., Rimington, C. and Goldberg, A. (1987). Porphyrins and Products of Haem Biosynthetic Pathway. In: *Disorders of Porphyrin Metabolism*. Plenum Publishing Corporation, New York, pp. 21-72.
- Moore, M. R. (1990). Historical introduction to porphyrins and porphyrias. In: *Biosynthesis of heme and chlorophyll*, Dailey, H. A., ed., McGraw-Hill Publishing Company; USA, pp. 1-54.
- Moran-Jiminez, M. J., Ged, C., Romana, M. et al (1996). Uroporphyrinogen decarboxylase: complete gene sequence and molecular study of three families with hepatoerythropoietic porphyria. *Amer. J. Hum. Genet.* 58: 712-721.
- Moreland, D. E., Blackmon, W. H., Todd, H. G. and Farmer, F. S. (1970). Effect of diphenylether herbicides on reactions of mitochondria and chloroplasts. *Weed Sci.* 18: 636-642.
- Morton, K. A., Kushner, J. P. and Burnham, B. F. (1981). Biosynthesis of porphyrins and heme from dioxoallevate from intact hepatocytes. *Proc. Natl. Acad. Sci. USA* 78: 5325.
- Muir, H. M. and Neuberger, A. (1950). The biogenesis of porphyrins. 2. The origin of the methyne carbon atoms. *Biochem. J.* 47: 97-104.
- Mulder, G. H. (1844). Über eisenfreies Hamatin. *J. Prakt. Chem.* 32: 186-197.
- Murkeji, S. K. and Pimstone, N. R. (1987). Evidence for two uroporphyrinogen decarboxylase isoenzymes in human erythrocytes. *Biochem. Biophys. Res. Commun.* 146(3): 1196-1203.

- Murkeji, S. K. and Pimstone, N. R. (1992). Uroporphyrinogen decarboxylase from human erythrocytes: purification, complete separation and partial characterization of two isoenzymes. *Intern. J. Biochem.* 24 (1): 105-119.
- Murphy, G. M., Hawk, J. L. M., Magnus, I. A., Barret, D. F., Elder, G. H. and Smith, S. G. (1986). Homozygous variegate porphyria: Two similar cases in unrelated families. *J. Roy. Soc. Med.* 79: 361-363.
- Mustajoki, P., Tenhunen, R., Niemi, K. A., Nordmann, Y., Kaariainen, H. and Norio, R. (1987). Homozygous variegate porphyria: a severe skin disease of infancy. *Clin. Genet.* 32: 300-305.
- Nakahashi, Y., Taketani, S., Okuda, M., Inoue, K. and Tokunaga, R. (1990). Molecular cloning and sequence analysis of cDNA encoding human ferrochelatase. *Biochem. Biophys. Res. Commun.* 173 (2): 748-755.
- Nakayashiki, T., Nishimura, K. and Inokushi, H. (1995). Cloning and sequencing of a previously unidentified gene that is involved in the biosynthesis of heme in *Escherichia coli*. *Gene* 153: 67-70.
- Namba, H., Narahara, K., Tsuji, X., Yokayama, Y. and Seino, Y. (1991). Assignment of human PBGD to 11q24.1 → 11q24.2 by *in situ* hybridisation and gene dosage studies. *Cytogenet. Cell. Genet.* 57:105-108.
- Nandi, D. L., Baker-Cohen, K. F. and Shemin, D. (1968). Delta-aminolevulinic acid dehydratase of *Rhodopseudomonas spheroides*: 3. Mechanism of porphobilinogen synthesis. *J. Biol. Chem.* 243 (6): 1224-1230.
- Nandihalli, U. B., Duke, M. V. and Duke, S. O. (1992). Quantitative structure-activity relationships of protoporphyrinogen oxidase-inhibiting diphenyl ether herbicides. *Pesticide Biochem. Physiol.* 43: 193-211.
- Nandihalli, U. B., Duke, M. V., Ashmore, J. W., Musco, V. A., Clark, R. D. and Duke, S. O. (1994). Enantioselectivity of protoporphyrinogen oxidase-inhibiting herbicides. *Pestic. Sci.* 40: 265-277.
- Narita, S., Tanaka, R., Ito, T., Okada, K., Taketani, S. and Inokuchi, H. (1996). Molecular cloning and characterization of a cDNA that encodes protoporphyrinogen oxidase of *Arabidopsis thaliana*. *Gene* 182: 169-175.
- Neidle, E. L. and Kaplan, S. (1993). Expression of the *Rhodobacter sphaeroides* *hemA* and *hemT* genes, encoding two 5-aminolevulinic acid synthase isozymes. *J. Bacteriol.* 175 (8): 2292-2303.
- Nencki, M. and Sieber, N. (1888). Über das Hamatoporphyrin. *Arch. Exp. Pathol. Pharmacol.* 24: 430-446.
- Neuberger, A. and Scott, J. J. (1953). Aminolaevulinic acid and porphyrin synthesis. *Nature* 172: 1093-1094.
- Nishimura, K., Nakayashiki, T. and Inokuchi, H. (1995a). Cloning and identification of the *hemG* gene encoding protoporphyrinogen oxidase (PPO) of *Escherichia coli* K-12. *DNA Res.* 2 (1): 1-8.

- Nishimura, K., Taketani, S. and Inokuchi, H. (1995b). Cloning of a human cDNA for protoporphyrinogen oxidase by complementation *in vivo* of a *hemG* mutant of *Escherichia coli*. J. Biol. Chem. 270 (14): 8076-8080.
- Nordmann, T. and Deybach, J-C. (1982). Congenital erythropoietic porphyria. Semin. Liv. Dis. 2: 154-163.
- Nordmann, Y. and Deybach, J-C. (1990). Human Hereditary Porphyria. In: Biosynthesis of heme and chlorophylls, Dailey, H. A. ed., McGraw-Hill, New York, 491-542.
- Nordmann, Y., de Verneuil, H., Deybach, J-C., Dafau, M. H. and Grandchamp, B. (1990). Molecular genetics of the porphyrias. Ann. Med. 22: 387-391.
- Nordmann, Y., Grandchamp, B., de Verneuil, H., Phung, N., Cartigny, B. and Fontaine, G. (1983). Harderoporphyria - a rare variant of hereditary coproporphyria. J. Clin. Invest. 72: 1139-1149.
- Norris, P. G., Elder, G. H. and Hawk, J. L. M. (1990). Homozygous variegate porphyria: a case report. Brit. J. Dermatol. 122: 253-257.
- Norton, E., Sarwar, M. and Shoolingin-Jordan, P. (1998). Mechanistic studies on *Escherichia coli* aminolaevulinic acid dehydratase. Biochem. Soc. Trans. 26: S285.
- O'Donnell, V. B., Smith, G. C. and Jones, O. T. (1994). Involvement of phenyl radicals in iodonium inhibition of flavoenzymes. Mol. Pharmacol. 46 (4): 778-785.
- Oh-hama, T., Seto, H., Otake, N. and Miyachi, S. (1982). ¹³C-NMR evidence for the pathway of chlorophyll biosynthesis in green algae. Biochem. Biophys. Res. Commun. 105 (2): 647-652.
- Orr, G. L. and Hess, F. D. (1981). Characterization of herbicidal injury by acifluorfen-methyl in excised cucumber (*Cucumis sativus* L.) cotyledons. Pestic. Biochem. Physiol. 16: 171-178.
- Orr, G. L. and Hogan, M. E. (1985). UV-A photo-oxidation of beta-carotenes in Triton X-100 micelles by nitrodiphenyl ether herbicides. J. Agric. Food Chem. 33: 968-972.
- Parkin, D. T. (1979). Molecular evolution, Chap. 3. In: An introduction to evolutionary genetics. Edward Arnold Publishers Ltd, 1st ed., London, pp. 50-74.
- Patton, G. M. and Beattie, D. S. (1973). Studies on hepatic δ -aminolevulinic acid synthetase. J. Biol. Chem. 248: 4467-4474.
- Pearson, W. R. and Lipman, D. J. (1988). Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci., USA, 85: 2444-2448.
- Perotti, M. E., Gavazzi, E., Trussardo, L., Margaretti, N. and Curti, B. (1987). Immunoelectron microscopic localization of D-amino acid oxidase in rat kidney and liver. Histochem. J. 19: 157-169.
- Phillips, J. D., Whitby, F. G., Kushner, J. P. and Hill, C. P. (1997). Characterization and crystallization of human uroporphyrinogen decarboxylase. Prot. Sci. 6 (6): 1343-1346.
- Pimstone, N. R. (1982). Porphyria cutanea tarda. Semin. Liv. Dis. 2: 132-142.
- Plewinska, M., Thunell, S., Holmberg, L., et al (1991). δ -aminolevulinic acid dehydratase deficient porphyria: identification of the molecular lesions in a severely affected homozygote. Amer. J. Genet. 49: 167-174.

- Poh-Fitzpatrick, M. B. (1980). A plasma porphyrin fluorescence marker for variegate porphyria. *Arch. Dermatol.* 116: 543-547.
- Poh-Fitzpatrick, M. B. (1985). Porphyrin-sensitized cutaneous photosensitivity: pathogenesis and treatment. *Clin. Dermatol.* 3: 41-82.
- Poh-Fitzpatrick, M. P. (1998). Clinical features of the porphyrias. *Clin. Dermatol.* 16(2): 251-264.
- Pollack, J. M. and van Noorden, S. (1987). *An Introduction to Immunocytochemistry – current techniques and problems.* Oxford University Press, Royal Microscopic Society.
- Ponka, P. (1997). *Blood* 89: 1-25.
- Pornprom, T., Matsumoto, H., Usui, K. and Ishizuka, K. (1994). Characterization of oxyfluorfen tolerance in selected soybean line. *Pestic. Biochem. Physiol.* 50: 107-114.
- Porra, R. J. and Falk, J. E. (1964). The enzymic conversion of coproporphyrinogen III into protoporphyrin IX. *Biochem. J.* 90: 69-75.
- Porra, R. J., Klein, O. and Wright, P. E. (1983). The proof by ^{13}C -NMR spectroscopy of the predominance of the C5 pathway over the Shemin pathway in chlorophyll biosynthesis in higher plants, and of the formation of the methyl ester group of chlorophyll from glycine. *Eur. J. Biochem.* 130 (3): 509-516.
- Poulson, R. (1976). The enzymic conversion of protoporphyrinogen IX to protoporphyrin IX in mammalian mitochondria. *J. Biol. Chem.* 251: 3730-3733.
- Poulson, R. and Polglase, W. J. (1974). Aerobic and anaerobic coproporphyrinogenase activity in extracts from *Saccharomyces cerevisiae* - Purification and characterization. *J. Biol. Chem.* 249: 6367-6372.
- Poulson, R. and Polglase, W. J. (1975). The enzymic conversion of protoporphyrinogen IX to protoporphyrin IX. *J. Biol. Chem.* 250: 1269-1274.
- Prasad, A. R. K. and Dailey, H. A. (1995). Generation of resistance to the diphenyl ether herbicide acifluorfen by MEL cells. *Biochem. Biophys. Res. Commun.* 215 (1): 186-191.
- Pretorius, C., du Toit, J. J., du Preez, J. C. and Meissner, P. N. (1997). Diagnosis of variegate porphyria at birth by demonstration of the R59W mutation. *S. Afr. Med. J.* 87 (6): 763, 765.
- Pritchard, M. K., Warren, G. F. and Dilley, R. A. (1980). Site of action of oxyfluorfen. *Weed Sci.* 28: 640-645.
- Proceedings of the International Conference on the Porphyrrias (1963). *S. Afr. Lab. Clin. Med.* 9: Special issue.
- Proulx, K. L. and Dailey, H. A. (1992). Characteristics of murine protoporphyrinogen oxidase. *Prot. Sci.* 1: 801-809.
- Puy, H., Deybach, J. C., Lamoril, J., et al (1997). Molecular epidemiology and diagnosis of acute intermittent porphyria. *Amer. J. Hum. Genet.*
- Puy, H., Robreau, A. M., Rosipal, R., Nordmann, Y. and Deybach, J. C. (1996). Protoporphyrinogen oxidase: complete genomic sequence and polymorphisms in the human gene. *Biochem. Biophys. Res. Commun.* 226 (1): 226-230.

- Ragan, C. I. and Bloxham, D. P. (1977). Specific labelling of a constituent polypeptide of bovine heart mitochondrial reduced nicotinamide-adenine dinucleotide-ubiquinone reductase by the inhibitor diphenyleneiodonium. *Biochem. J.* 163 (3): 605-615.
- Randolph-Anderson, B. L., Sato, R., Johnson, A. M., Harris, E. H., Hauser, R. C., Oeda, K., Ishige, F., Nishio, S., Gillham, N. W. and Boynton, J. E. (1998). Isolation and characterisation of mutant protoporphyrinogen oxidase gene from *Chlamydomonas reinhardtii* conferring resistance to porphyrin herbicides. *Plant Mol. Biol.* 38: 839-859.
- Reddy, K. N., Dayan, F. E. and Duke, S. O. (1997). QSAR analysis of protoporphyrinogen oxidase inhibitors. *Pestic. Biochem. Biophys.* 43: 193-211.
- Reddy, K. N., Nandihalli, U. B., Lee, H. J., Duke, M. V. and Duke, S. O. (1995). Predicting activity of protoporphyrinogen oxidase inhibitors by computer-aided molecular modelling. *Amer. Chem. Soc. Symposium Series* 589: 221-224.
- Retzlaff, K. and Boger, P. (1996). An endoplasmic reticulum plant enzyme has protoporphyrinogen oxidase activity. *Pestic. Biochem. Physiol.* 54: 105-114.
- Ridley, S. M. (1983). Interaction of chloroplasts with inhibitors. Effects of two diphenylether herbicides, fomesafen and nitrofluorfen, on electron transport, and some comparisons with dibromothiquinone, diuron and paraquat. *Plant Physiol.* 72: 461-468.
- Rimington, C. (1939). A re-investigation of turacin, the copper porphyrin pigment of certain birds of the *Musophagidae*. *Proc. Roy. Soc. Br.* 127: 106-120.
- Rimington, C. (1985). A review of the enzymic errors in the various porphyrias. *Scand. J. Clin. Lab. Invest.* 45: 291-301.
- Rio, B., Parrent-Massin, D. and Hoellinger, H. (1997). Effects of a diphenyl-ether herbicide, oxyfluorfen, on human BFU-E/CU-E development and haemoglobin synthesis. *Hum. Exper. Toxicol.* 16: 115-122.
- Roberts, A. G. and Elder, G. H. (1997). Purification and properties of uroporphyrinogen decarboxylase from human erythrocytes. *Methods Enzymol.* 281: 349-355.
- Roberts, A. G., Elder, G. H., De Salamanca, R. E., and Mascaro, J. M. (1995a). A mutation (G281E) of the human uroporphyrinogen decarboxylase gene causes both hepatoerythropoietic porphyria and familial porphyria cutanea tarda. *J. Invest. Dermatol.* 104: 500-502.
- Roberts, A. G., Puy, H., Dailey, T. A., Morgan, R. R., Whatley, S. D., Dailey, H. A., Martasek, P., Nordmann, Y., Deybach, J-C. and Elder, G. H. (1998). Molecular characterization of homozygous variegate porphyria. *Hum. Mol. Genet.* 7 (12): 1921-1925.
- Roberts, A. G., Whatley, S. D., Dailey, T. A., Dailey, H. A. and Elder, G. H. (1996). Identification of a mutation in the protoporphyrinogen oxidase gene in homozygous variegate porphyria. *Hepatology* 23: 309.

- Roberts, A. G., Whatley, S. D., Daniels, J., Holmans, P., Fenton, I., Owen, M. J., Thompson, P., Long, C. and Elder, G. H. (1995b). Partial characterization and assignment of the gene for protoporphyrinogen oxidase and variegate porphyria to human chromosome 1q23. *Hum. Molec. Genet.* 4(12): 2387-2390.
- Romana, M., Dubart, A., Beaupain, D., Chabret, C., Goossens, M. and Romeo, P. H. (1987b). Structure of the gene for human uroporphyrinogen decarboxylase. *Nucl. Acids Res.* 15 (18): 7343-7355.
- Romana, M., Le Boulch, R. and Romeo P. H. (1987a). Rat uroporphyrinogen decarboxylase cDNA: nucleotide sequence and comparison to human uroporphyrinogen decarboxylase. *Nucl. Acid Res.* 15 (17): 7211.
- Romeo, G. and Levin, E. (1969). Uroporphyrinogen-III cosynthase in human congenital erythropoietic porphyria. *Proc. Natl. Acad. Sci USA* 63: 856-863.
- Romeo, R. H., Raich, N., Dubart, A., Beaupain, D., Pryor, M., Kushner, J., Cohen-Solal, M. and Goossens, M. (1986). Molecular cloning and nucleotide sequence of a complete human uroporphyrinogen decarboxylase cDNA. *J. Biol. Chem.* 261 (21): 9825-9830.
- Roper, J. M. and Smith, A. G. (1997). Molecular localisation of ferrochelatase in higher plant chloroplasts. *Eur. J. Biochem.* 246 (1): 32-37.
- Rossi, E., Attwood, P. V. and Garcia-Webb, P. (1992). Inhibition of human lymphocyte coproporphyrinogen oxidase activity by metals, bilirubin and haemin. *Biochem. et Biophys. Acta* 1135: 262-268.
- Rufenacht, U. B., Schneider-Yin, X., Schafer, B. W., Taketani, S., Deybach, J-C. and Minder, E. I. (1998). Rapid molecular diagnosis of erythropoietic protoporphyria among Swiss patients. *Clin. Chem. Lab. Med.* 36 (10): 763-765.
- Runge, W. and Watson, C. J. (1962). Experimental production of skin lesions in human cutaneous porphyria. *Proc. Sci. Exp. Biol. Med.* 109: 809-811.
- Saillet, H. (1896). De l'urospectrine. *Rev. Med.* 16: 542-552.
- Sancovich, H. A., Batlle, A. M. del C. and Grinstein, M. (1969). Porphyrin synthesis. VI. Separation and purification of porphobilinogen deaminase and uroporphyrinogen isomerase from cow liver. Porphobilinogenase an allosteric enzyme. *Biochim. Biophys. Acta* 191: 130-143.
- Sano, S. and Granick, S. (1961). Mitochondrial coproporphyrinogen oxidase and protoporphyrin formation. *J. Biol. Chem.* 236: 1173-1180.
- Sarkany, R. P. E., and Cox, T. M. (1995). Autosomal recessive erythropoietic protoporphyria. A syndrome of photosensitivity and liver failure. *Quart. J. Med.* 88: 541-549.
- Sarkin, L., Mendez, M., Rossetti, M. V., et al (1996). Identification of six new mutations in the uroporphyrinogen decarboxylase gene. *Amer. J. Hum. Genet.* 59: A285.
- Sasarman, A., Letowski, J., Czaika, G., Ramirez, V. Nead, M. A., Jacobs, J. M., and Morais, R. (1993). Nucleotide sequence of the *hemG* gene involved in the protoporphyrinogen oxidase activity of *Escherichia coli* K12. *Can. J. Microbiol.* 39: 1155-1161.

- Sasarman, A., Nepveu, A., Echelard, V., Demetryszyn, J., Drolet, M. and Goyer, C. (1987). Molecular cloning and sequencing of the *hemD* gene of *Escherichia coli* K12 and preliminary data on the Uro operon. *J. Bacteriol.* 169: 4257-4262.
- Sassa, S. (1998). ALAD porphyria. *Semin. Liv. Dis.* 18: 95-101.
- Sassa, S. and Kappas, A. (1983). Hereditary tyrosinemia and the heme biosynthetic pathway. Profound inhibition of δ -aminolevulinic acid dehydratase activity by succinylacetone. *J. Clin. Invest.* 71: 625-634.
- Sassa, S., Kondo, M., Taketani, S., Nomura, N., Furuyama, K., Akagi, R., Nagai, T., Terajima, M., Galbraith, R. and Fujita, H. (1997). Molecular defects of the coproporphyrinogen oxidase gene in hereditary coproporphyria. *Cell. Mol. Biol.* 43(1): 59-66.
- Sassa, S., Solish, G., Levere, R. D. and Kappas, A. (1975). Studies in porphyria IV. Expression of gene defect of acute intermittent porphyria in cultured human skin fibroblasts and amniotic cells: Prenatal diagnosis of the porphyric trait. *J. Exp. Med.* 142: 722-731.
- Sassa, S., Zalar, G. L. and Kappas, A. (1978). Studies in porphyria VII. Induction of uroporphyrinogen I synthase and expression of the gene defect of acute intermittent porphyria in mitogen stimulated human lymphocytes. *J. Clin. Invest.* 61: 499-508.
- Sato, R., Yamamoto, M., Shibata, H., Oshio, H., Harris, E. H., Gillham, N. W., and Boynton, J. E. (1994). Characterization of a mutant of *Chlamydomonas reinhardtii* resistant to protoporphyrinogen oxidase inhibitors. In: Duke, S. O. and Rebeiz, C. A. (eds), *Porphyric Pesticides: Chemistry, Toxicology and Pharmaceutical applications*. Amer. Chem. Soc. Symp. Ser. 559, Washington, DC. pp. 91-104.
- Scalla, R. and Matringe, M. (1994). Inhibitors of protoporphyrinogen oxidase as herbicides: diphenyl ethers and related photobleaching molecules. *Rev. Weed Sci.* 6: 103-132.
- Schmid, R. (1960). Cutaneous porphyria in Turkey. *N. Engl. J. Med.* 263: 397-398.
- Schmid, R. and Shemin, D. (1955). *J. Amer. Chem. Soc.* 77: 506-508.
- Schoenfeld, N., Mamet, R., Dotan, I, et al. (1995). Relation between uroporphyrin excretion, acute attacks of hereditary coproporphyria and successful treatment with haem arginate. *Clin. Sci.* 88: 365-369.
- Schoenhaut, D. S. and Curtis, P. J. C. (1986). Nucleotide sequence of mouse 5-aminolevulinic acid synthase cDNA and expression of its gene in hepatic and erythroid tissues. *Gene* 48 (1): 55-63.
- Scott, A. I., Townsend, C. A., Okada, K., Kajimara, M. and Cushley, R. J. (1972). Biosynthesis of corrinoids. Uroporphyrinogen III as a precursor of vitamin B12. *J. Amer. Chem. Soc.* 94: 8269-8271.
- Scotto, A. W., Chang, L. F. and Beattie, D. S. (1983). The characterisation and sub-mitochondrial localisation of delta-aminolevulinic acid synthase and an associated amidase in rat liver mitochondria using an improved assay for both enzymes. *J. Biol. Chem.* 258: 81-90.
- Seehra, J. S., Jordan, P. M. and Akhtar, M. (1982). Anaerobic and aerobic coproporphyrinogen III oxidases of *Rhodospseudomonas spheroides*. Mechanism and stereochemistry of vinyl group formation. *Biochem J.* 269 (3): 709-718.

- Seki, Y., Kawanishi, S. and Sano, S. (1986). Uroporphyrinogen decarboxylase purification from chicken erythrocytes. *Methods Enzymol.* 123: 415-421.
- Sellers, V. M., Johnson, M. K. and Dailey, H. A. (1996). Function of the [2Fe-2S] cluster in mammalian ferrochelatase: a possible role as a nitric oxide sensor. *Biochem.* 35 (8): 2699-2704.
- Sellers, V. M., Wang, K. F., Johnson, M. K. and Dailey, H. A. (1998). Evidence that the fourth ligand to the [2Fe-2S] cluster in animal ferrochelatase is a cysteine. Characterization of the enzyme from *Drosophila melanogaster*. *J. Biol. Chem.* 273 (35): 22311-22316.
- Senior, N. M., Brockenhurst, K., Cooper, J. B., Wood, S. P., Erskine, P., Shoolingin-Jordan, P. M., Thomas, P. G. and Warren, M. J. (1996). Comparative studies on the 5-aminolaevulinic acid dehydratases from *Pisum sativum*, *Escherichia coli* and *Saccharomyces cerevisiae*. *Biochem. J.* 320 (2): 401-412.
- Senior, N. M., Siligardi, G., Drake, A., Thomas, P. G. and Warren, M. J. (1997). Structural studies on 5-aminolaevulinic acid dehydratase from *Saccharomyces cerevisiae*. *Biochem. Soc. Trans.* 25 (1): 78S.
- Shemin, D and Wittenberg, J. (1951). The mechanism of porphyrin formation - the role of the tricarboxylic acid cycle. *J. Biol. Chem.* 192: 315.
- Shemin, D. (1976). 5-Aminolevulinate dehydratase - Structure, function and mechanism. *Phil. Trans. Roy. Soc. London B* 273: 109-115.
- Shemin, D. and Rittenberg, D. (1946). The biological utilisation of glycine for the synthesis of the protoporphyrin of hemoglobin. *J. Biol. Chem.* 166: 621-625.
- Shemin, D. and Russell, C. S. (1953). Delta-aminolevulinic acid, its role in the biosynthesis of porphyrins and purines. *J. Amer. Chem. Soc.* 75: 4873-4875.
- Shemin, D., Russell, C. S. and Abramsky, T. (1955). The succinate glycine cycle. I. The mechanism of pyrrole synthesis. *J. Biol. Chem.* 215: 613-620.
- Shibuya, H., Nonneman, D., Tamassia, M., Allphin, O. L. and Johnson, G. S. (1995). The coding sequence of the bovine ferrochelatase gene. *Biochem. et Biophys. Acta* 1231 (1): 117-120.
- Shoolingin-Jordan, P. M. (1998). Structure and mechanism of enzymes involved in the assembly of the tetrapyrrole macrocycle. *Biochem. Soc. Trans.* 26: 326-336.
- Shoolingin-Jordan, P. M., Warren, M. J. and Awan, S. J. (1996). Discovery that the assembly of the dipyrromethane cofactor of porphobilinogen deaminase holoenzyme proceeds initially by the reaction of preuroporphyrinogen with the apoenzyme. *Biochem. J.* 316 (2): 373-376.
- Shoolingin-Jordan, P. M., Warren, M. J. and Awan, S. J. (1997). Dipyrromethane cofactor assembly of porphobilinogen deaminase: formation of apoenzyme and preparation of holoenzyme. *Methods Enzymol.* 281: 317-327.
- Siepkner, L. J., Ford, M., de Kock, R., and Kramer, S. (1987). Purification of bovine protoporphyrinogen oxidase: immunological cross-reactivity and structural relationship to ferrochelatase. *Biochim. Biophys. Acta* 913: 349-358.

- Silbergeld, E. K. and Fowler, B. A. (1987). Mechanisms of chemical-induced porphyriopathies. *Ann. N. Y. Acad. Sci.* 514: 1-352.
- Smith, A. G. and Francis, J. E. (1981). Investigation of rat liver uroporphyrinogen decarboxylase - Comparison of porphyrinogens I and III as substrates and the inhibition by porphyrins. *Biochem J.* 195: 241-250.
- Smith, A. G., Marsh, O. and Elder, G. H. (1993). Investigation of the subcellular location of the tetrapyrrole-biosynthesis enzyme coproporphyrinogen oxidase in higher plants. *Biochem. J.* 292 (2): 503-508.
- Smith, K. M. eds (1975). *Porphyrins and metalloporphyrins*, Elsevier, Amsterdam, 1-754.
- Smith, S. G. (1986). Hepatoerythropoietic porphyria. *Semin. Dermatol.* 5: 125-137.
- Smythe, E. and Williams, D. C. (1988). Rat liver uroporphyrinogen III synthase has similar properties to the enzyme from *Euglena gracilis* including absence of a requirement for reversibly bound cofactor. *Biochem. J.* 253 (1): 275-279.
- Soret, J. (1883). Reserchers sur l'absorption des rayons ultra-violet par diverses substances. *Arch. Sci. Phys. Nat.* 10: 430-485.
- Sorkin, L., Mendez, M., Rosseti, M. V., et al (1996). Identification of six new mutations in the uroporphyrinogen decarboxylase gene. *Amer. J. Hum. Genet.* 59: A285.
- Spencer, P. and Jordan, P. M. (1993). Purification and characterization of 5-aminolaevulinic acid dehydratase from *Escherichia coli* and a study of the reactive thiols at the metal-binding domain. *Biochem J.* 290 (1): 279-287.
- Spencer, P. and Jordan, P. M. (1994a). Investigation of the nature of the two metal-binding sites in 5-aminolaevulinic acid dehydratase from *Escherichia coli*. *Biochem. J.* 300: 373-381.
- Spencer, P. and Jordan, P. M. (1994b). 5-aminolaevulinic acid dehydratase: characterization of the alpha and beta metal-binding sites of the *Escherichia coli* enzyme. *Ciba-Foundation Symposium* 180: 50-64, discussion 64-69.
- Spencer, P. and Jordan, P. M. (1995). Characterisation of the two 5-aminolaevulinic acid binding sites, the A- and P-sites, of 5-aminolaevulinic acid dehydratase from *Escherichia coli*. *Biochem. J.* 305 (1): 151-158.
- Spivey, A. C., Capretta, A., Frampton, C. S., et al (1996). Biosynthesis of porphyrins and related macrocycles. Part 45. Determination by a novel X-ray method of the absolute configuration of the spiro lactam which inhibits uroporphyrinogen III synthase (cosynthase). *J. Chem. Soc. Perkin Trans 1*: 2091-2102.
- Srivastava G, Borthwick I. A., Macguire, D. J., et al (1988). Regulation of 5-aminolevulinate acid synthase mRNA in different rat tissues. *J Biol Chem.* 263: 5202-5209.

- Srivastava, G., Borthwick, I. A., Brooker, J. D., Wallace, J. C., May, B. K. and Elliot, W. H. (1983). Hemin inhibits transfer of pre-5-aminolaevulinate synthase into chick liver mitochondria. *Biochem. Biophys. Res. Commun.* 117: 344-349.
- Stamford, N. P., Capretta, A. and Battersby, A. R. (1995). Expression, purification and characterisation of the product from the *Bacillus hemD* gene, uroporphyrinogen III synthase. *Eur. J. Biochem.* 231 (1): 236-241.
- Stein, J. A. and Tschudy, D. P. (1970). Acute intermittent porphyria: a clinical and biochemical study of 46 patients. *Medicine* 49: 1-16.
- Stoltz, M. and Dornemann, D. (1996). Purification, metal cofactor, N-terminal sequence and subunit composition of a 5-aminolevulinic acid dehydratase from the unicellular green alga *Scenedesmus obliquus*, mutant C-2A'. *Eur. J. Biochem.* 236 (2): 600-608.
- Straka, J. G. and Kushner, J. P. (1983). Purification and characterization of bovine hepatic uroporphyrinogen decarboxylase. *Biochem.* 22: 4664-4672.
- Strand, L. J., Meyer, U. A., Felsher, B. F., Redeker, A. G. and Marver, H. S. (1972). Decreased red cell uroporphyrin-I synthetase activity in intermittent acute porphyria. *J. Clin. Invest.* 51: 2530-2536.
- Strierle, A., Upadhyay, R. and Strobel, G. (1991). Cyperin, a phytotoxin produced by *Aschochyta cypericola*, a fungal pathogen of *Cyperus rotundus*. *Phytochemistry* 30: 2191-2192.
- Stuart, R. A. and Neupert, W. (1996). Topogenesis of inner membrane proteins of mitochondria. *Trends Biochem. Sci.* 21 (7): 261-267.
- Sturrock, E. D., Meissner, P. N., Maeder, D. L. and Kirsch, R. E. (1989). Uroporphyrinogen decarboxylase and protoporphyrinogen oxidase in dual porphyria. *S Afr. Med. J.* 76 (8): 405-408.
- Subbiah, S. and Harrison, S. C. (1989). A method for multiple sequence alignment with gaps. *J. Mol. Biol.* 209: 539-548.
- Susa, S., Daimon, M., Yamamori, I., Kondo, M., Yamatani, K., Sasaki, H. and Kato, T. (1998). A novel mutation of coproporphyrinogen oxidase (CPO) gene in a Japanese family. *J. Hum. Genet.* 43(3): 182-184.
- Swofford, D. L. and Olsen, G. J. (1996). Phylogenetic Inference, Chap. 11. In: *Molecular systematics*, Hillis, D. M., Moritz, C. and Mable, B. K. eds, Sinauer Associates, Inc., Sunderland, MA, pp. 407-514.
- Tait, G. H. (1969). Coproporphyrinogenase activity in extracts from *Rhodopseudomonas spheroides*. *Biochem. Biophys. Res. Commun.* 37: 116-122.
- Tait, G. H. (1972). Coproporphyrinogenase activities in extracts of *Rhodopseudomonas spheroides* and *Chromatium* strain D. *Biochem. J.* 128: 1159-1169.
- Taketani, S. and Tokunaga, R. (1981). Rat liver ferrochelatase. Purification, properties, and stimulation by fatty acids. *J. Biol. Chem.* 256 (24): 12748-12753.
- Taketani, S. and Tokunaga, R. (1982). Purification and substrate specificity of bovine liver ferrochelatase. *Eur. J. Biochem.* 127: 443-447.

- Taketani, S., Inazawa, J., Abe, T., Furukawa, T., Kohno, H., Tokunaga, R., Nishimura, K. and Inokuchi, H. (1995b). The human protoporphyrinogen oxidase gene (PPOX): organisation and localisation to chromosome 1. *Genomics* 29: 698-703.
- Taketani, S., Inazawa, J., Nakahashi, Y., Abe, T. and Tokunaga, R. (1992). Structure of the human ferrochelatase gene. Exon/intron gene organisation and location of the gene to chromosome 18. *Eur. J. Biochem.* 205 (1): 217-222.
- Taketani, S., Kohno, H., Furukawa, T., Yoshinaga, T. and Tokunaga, R. (1994). Molecular cloning, sequencing and expression of cDNA encoding human coproporphyrinogen oxidase. *Biochem. Biophys. Acta* 1183 (3): 547-549.
- Taketani, S., Nakahashi, Y., Osumi, T. and Tokunaga, R. (1990). Molecular cloning, sequencing and expression of mouse ferrochelatase. *J. Biol. Chem.* 265 (32): 19377-19380.
- Taketani, S., Yoshinaga, T., Furukawa, T., Kohno, H., Tokunaga, R., Nishimura, K. and Inokuchi, H. (1995a). Induction of terminal enzymes for heme biosynthesis during differentiation of mouse erythroleukemia cells. *Eur. J. Biochem.* 230: 760-765.
- Tew, D. G. (1993). Inhibition of cytochrome P450 reductase by the diphenyleneiodonium cation. Kinetic analysis and covalent modification. *Biochem.* 32 (38): 10209-10215.
- The QIA Expressionist (1992). The high level expression and protein purification system. 2nd ed., Qiagen Ltd, Germany, pp 51.
- Thomas, S. D. and Jordan, P. M. (1986). Nucleotide sequence of the *hemC* locus encoding porphobilinogen deaminase of *Escherichia coli* K12. *Nucl. Acid Res.* 14: 6215-6226.
- Thompson, R. P. H., Molland, E. A., Nicholson, D. C. and Gray, C. H. (1973). Erythropoietic protoporphyria and cirrhosis in sisters. *Gut* 14: 934-938.
- Thudicum, J. D. W. (1867). Report on Researches Intended to Promote an Improved Chemical Identification of Disease, 10th Report of the Medical Officer, Privy Council, Appendix 7, H. M. S. O., London, pp. 152-233.
- Timonen, K., Niemi, K. M., Mustajoki, P and Tenhunen, R. (1990). Skin changes in variegate porphyria. Clinical, histopathological and ultrastructural study. *Arch. Dermatol. Res.* 282: 108-114.
- Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76: 4350-4354.
- Towers, G. H. N. and Arnason, J. T. (1988). Photodynamic herbicides. *Weed Technol.* 2: 545-549.
- Troup, B., Hungerer, C. and Jahn, D. (1995). Cloning and characterization of the *Escherichia coli hemN* gene encoding the oxygen-independent coproporphyrinogen III oxidase. *J. Bacteriol.* 177: 3326-3331.
- Troup, B., Jahn, M., Hungerer, C. and Jahn, D. (1994). Isolation of the *hemF* operon containing the gene for the *Escherichia coli* aerobic coproporphyrinogen III oxidase by *in vivo* complementation of a yeast *HEM13* mutant. *J. Bacteriol.* 176: 673-680.

- Tsai, S-F., Bishop, D. F. and Desnick, R. J. (1987). Purification and properties of uroporphyrinogen III synthase from human erythrocytes. *J. Biol. Chem.* 262 (3): 1268-1273.
- Tsai, S-F., Bishop, D. and Desnick, R. (1988). Human uroporphyrinogen III synthase: molecular cloning, nucleotide sequence, and expression of a full-length cDNA. *Proc. Natl. Acad. Sci. USA* 85 (19): 7049-7053.
- Tschudy, D. P. (1980). Porphyrin Metabolism and the Porphyrrias,. In: *Metabolic disease and control*, Bondy, P. K. and Rosenber, L. E., eds. Philadelphia, W. B. Saunders, pp. 939-1007.
- Tsukamoto, I., Yoshinaga, T. and Sano, S. (1980). Zinc and cysteine residues in the active site of bovine liver δ -aminolevulinic acid dehydratase. *Int. J. Biochem.* 12: 751-756.
- Urban-Grimal, D., Volland, C., Garnier, T., Dehoux, P. and Labbe-Bois, R. (1986). The nucleotide sequence of the *HEMI* gene and evidence for a precursor form of the mitochondrial 5-aminolevulinate synthase in *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 156 (3): 511-519.
- Varsano, R., Matringe, M., Magnin, N., Mornet, R. and Scalla, R. (1990). Competitive interaction of three peroxidizing herbicides with the binding of [3 H]acifluorfen to corn etioplast membranes. *Febs Lett.* 272 (1, 2): 106-108.
- Vartikovski, L., Kushner, J. P. and Burnham, B. F. (1980). Biosynthesis of porphyrin precursors - purification and characterization of mammalian L-alanine-dioxovaleric acid aminotransferase. *J. Biol. Chem.* 355: 3742.
- Venkatasubbaiah, P., Van Dyke, C. G. and Chilton, W. S. (1992). Phytotoxic metabolites of *Phoma sorghina*, a new foliar pathogen of pokeweed. *Mycologia* 84: 715-723.
- Verkamp, E. and Chelm, B. K. (1989). Isolation, nucleotide sequence and preliminary characterization of the *Escherichia coli* K-12 *hemA* gene. *J. Bacteriol.* 171 (9): 4728-4735.
- Viljoen, D. J., Cummins, R., Alexopoulos, J. and Kramer, S. (1983). Protoporphyrinogen oxidase and ferrochelatase in variegate porphyria. *Eur. J. Clin. Invest.* 13: 283-287.
- Volland, C. and Felix, F. (1984). Isolation and properties of 5-aminolevulinate synthase from the yeast *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 142 (3): 551-557.
- Waldenstrom, J. (1937). Studien uber porphyrie. *Acta. Med. Scand.* 92 (Suppl.): 1-254.
- Waldenstrom, J. (1957). The porphyrias as inborn errors of metabolism. *Amer. J. Med.* 22: 758-773.
- Wang, W-Y., Gough, S. P. and Kannangara, C. G. (1981). *Carlsberg Res. Comm.* 46: 243-257.
- Ward, patent number WO 95/34659 (1995). Cited from Johnston et al, 1998.
- Warnich, L., Kotze, M. J., Groenewald, I. M., Groenewald, J. Z., van Brakel, M. G., van Heerden, C. J. et al (1996a). Identification of three mutations and associated haplotypes in the protoporphyrinogen oxidase gene in South African families with variegate porphyria. *Hum. Molec. Genet.* 5 (7): 981-984.
- Warnich, L., Meissner, P. N., Hift, R. J., Louw, J. H., van Heerden, C. J. and Retief, A. E. (1996b). Mapping of the variegate porphyria (VP) gene: contradictory evidence for linkage between VP and microsatellite markers at chromosome 14q32. *Hum. Genet.* 97: 690-692.

- Warnick, G. R. and Burnham, B. F. (1971). Regulation of porphyrin biosynthesis. Purification and characterization of 5-aminolevulinic acid synthase. *J. Biol. Chem.* 246 (22): 6880-6885.
- Watanabe, W., Che, F.-S., Iwano, M., Takayama, S., Nakano, T., Yoshida, S., and Isogai, A. (1998). Molecular characterisation of photomixotrophic tobacco cells resistant to protoporphyrinogen oxidase-inhibiting herbicides. *Plant Physiol.* 118: 751-758.
- Weber, H. A. and Gloer, J. B. (1988). Interference competition among natural fungal competitors: An antifungal metabolite from the coprophilus fungus *Preussia fleishhakkii*. *J. Natural Products* 51: 879-883.
- Went, L. N. and Klasen, E. C. (1984). Genetic aspects of erythropoietic protoporphyria. *Ann. Hum. Genet.* 48: 105-117.
- Westall, R. G. (1952). Isolation of porphobilinogen from the urine of a patient with acute porphyria. *Nature* 170: 614.
- Weston, P. H. (1994). Methods for Rooting Cladistic Trees. In: *Models in phylogeny reconstruction*, Scotland, R. W., Siebert, D. J. and Williams, D. M. eds, Oxford University Press, Oxford, pp. 125-155.
- Wetmur, J. G., Bishop, D., Cantelmo, C. and Desnick, R. J. (1986). Human δ -aminolevulinate dehydratase: nucleotide sequence of a full-length cDNA clone. *Proc. Natl. Acad. Sci. USA* 83: 7703-7707.
- Whatley, S. D., Puy, H., Morgan, R. R., Robreau, A. G., Nordmann, Y., Elder, G. H. and Deybach, J.-C. (1999). Variegate porphyria in Western Europe: Identification of PPOX gene mutations in 104 families, extent of allelic heterogeneity, and absence of correlation between phenotype and type of mutation. *Am. J. Hum. Genet.* 65(4): 984-994.
- Whitby, F. G., Phillips, J. D., Kushner, J. P. and Hill, C. P. (1998). Crystal structure of human uroporphyrinogen decarboxylase. *EMBO. J.* 17 (9): 2463-2471.
- Whitcombe, D. M., Carter, N. R., Albertson, D. G., Smith, S. J., Rhodes, D. A. and Cox, T. M. (1991). Assignment of the human ferrochelatase gene (FECH) and a locus for protoporphyria to chromosome 18q22. *Genomics* 11 (4): 1152-1154.
- Whiting, M. J. and Elliott, W. H. (1972). Purification and properties of solubilized mitochondrial δ -aminolevulinic acid synthetase and comparison with the cytosol enzyme. *J. Biol. Chem.* 247: 6818-6826.
- Wierenga, R. K., Tepstra, P. and Hol, W. J. J. (1986). Prediction of the occurrence of the ADP-binding $\beta\alpha\beta$ -fold in proteins, using an amino acid sequence fingerprint. *J. Mol. Biol.* 187: 101-107.
- Witkowski, D. A. and Halling, B. P. (1988). Accumulation of photodynamic tetrapyrroles induced by acifluorfen-methyl. *Plant Physiol.* 87: 632-637.
- Witkowski, D. A. and Halling, B. P. (1989). Inhibition of plant protoporphyrinogen oxidase by the herbicide acifluorfen-methyl. *Plant Physiol.* 90: 1239-1242.
- Wu, C., Xu, W., Kozak, C. A. and Desnick, R. J. (1996). Mouse uroporphyrinogen decarboxylase: cDNA cloning, expression, and mapping. *Mammalian Genome* 7 (5): 349-352.

- Wu, W. H., Shemin, D., Richards, K. E. and Williams, R. C. (1974). The quaternary structure of δ -aminolevulinate dehydratase from bovine liver. *Proc. Natl. Acad. Sci. USA* 71: 1767-1770.
- Wyckoff, E. E., Phillips, J. D., Sowa, A. M., Franklin, M. R. and Kushner, J. P. (1996). Mutational analysis of human uroporphyrinogen decarboxylase. *Biochim. Biophys. Acta* 1298: 294-304.
- Xu, K. and Elliot, T. (1993). An oxygen-dependent coproporphyrinogen oxidase encoded by the *hemF* gene of *Salmonella typhimurium*. *J. Bacteriol.* 175 (16): 4990-4999.
- Xu, K. and Elliot, T. (1994). Cloning, DNA sequence, and complementation analysis of the *Salmonella typhimurium hemN* gene encoding a putative oxygen-independent coproporphyrinogen III oxidase. *J. Bacteriol.* 176 (11): 3196-3203.
- Xu, W., Kozak, C. A. and Desnick, R. J. (1995). Uroporphyrinogen III synthase: molecular cloning, nucleotide sequence, expression of a mouse full-length cDNA, and its localisation on mouse chromosome 7. *Genomics* 26 (3): 556-562.
- Yamamoto M., Kure S. and Engel J. D. (1988). Structure, turnover, and heme-mediated suppression of the level of mRNA encoding rat liver delta-aminolevulinate synthase. *J. Biol. Chem.* 263: 15973-15979.
- Yamamoto, M., Yew, N. S., Federspiel, M., Dodgson, J. B., Hayashi, N. and Engel, J. D. (1985). Isolation of recombinant cDNAs encoding chicken erythroid 5-aminolevulinate synthase. *Proc. Natl. Acad. Sci. USA* 82 (11): 3702-3706.
- Yamauchi, K., Hayashi, N. and Kikuchi, G. (1980). Translocation of δ -aminolevulinate synthetase from the cytosol to the mitochondria and its regulation by hemin in the rat liver. *J. Biol. Chem.* 255: 1746-1751.
- Yomogida, K., Yamamoto, M., Yamagami, T., Fjita, H. and Hayashi, N. (1993). Structure and expression of the gene encoding rat nonspecific form delta-aminolevulinate synthase. *J. Biol. Chem.* 113 (3): 364-371.
- Yoshinaga, T. and Sano, S. (1980a). Coproporphyrinogen oxidase. II: Reaction mechanism and role of tyrosine residues on the activity. *J. Biol. Chem.* 255 (10): 4722-4726.
- Yoshinaga, T. and Sano, S. (1980b). Coproporphyrinogen oxidase. I: Purification, properties and activation by phospholipids. *J. Biol. Chem.* 255 (10): 4727-4731.
- Zagorec, M., Buhler, J. M., Treich, I., Keng, T., Guarente, L. and Labbe-Bois, R. (1988). Isolation, sequence and regulation by oxygen of the yeast *HEM13* gene coding for coproporphyrinogen oxidase. *J. Biol. Chem.* 263 (20): 9718-9724.